INDIGENOUS *SALVIA* SPECIES − AN INVESTIGATION OF THEIR PHARMACOLOGICAL ACTIVITIES AND PHYTOCHEMISTRY

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Declaration

I, Guy Paulin Poungoué Kamatou, declare that this thesis is my own work except where acknowledged. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

……..day of ……...….2006

This thesis is dedicated to my Lord Jesus Christ and the family Poungoué for their love and support.

Publications Arising from this Study

Kamatou, G.P.P., Viljoen, A.M., Gono-Bwalya, A.B., van Zyl, R.L., van Vuuren , S.F., Lourens, A.C.U., Başer, K.H.C., Demirci, B., Lindsey, K. L., van Staden, J., Steenkamp, P. 2005. The *in vitro* pharmacological activities and a chemical investigation of three South African *Salvia* species. *Journal of Ethnopharmacology* **102,** 382–390 (Abstract in Appendix A).

Kamatou, G.P.P., Viljoen, A.M., van Zyl, R.L., van Vuuren, S.F., Figueiredo, A.C., Barroso, J.G., Pedro, L.G., Tilney, P.M. 2006. Chemical composition, leaf trichome types and biological activities of essential oils of four related *Salvia* species indigenous to South Africa. *Journal of Essential Oil Research* **18,** 72–79 (Abstract in Appendix A).

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Conferences/presentations

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Abstract

The genus *Salvia* belongs to the family Lamiaceae and encompasses 900 species worldwide of which 26 are found in southern Africa and many of them are used in local traditional medicine. However, the phytochemistry and pharmacological activities of the South African species have not been extensively investigated.

The leaf trichome morphology that may be used to distinguish species was investigated with the scanning electron and light microscopy. Both glandular (capitate or peltate) and non-glandular trichomes were identified in all species.

The essential oils were isolated by hydro-distillation and analysed by GC and GC-MS methods. The oil yield was relatively low and ranged from 0.004 (*S. radula*) to 0.50% (*S. muirii*) (w/w). Major components identified include α-pinene, 1,8-cineole, linalool, limonene, myrcene, β-caryophyllene, spathulenol, β-caryophyllene oxide, viridiflorol, δ-3-carene and α-bisabolol. High performance liquid chromatography analysis was used to identify phenolic compounds in 17 solvent extracts. Betulafolientriol oxide was detected in all species. Rosmarinic acid was only absent in *S. verbenaca*, while *S. garipensis* and *S. radula* were the only species which lacked oleanolic acid/ursolic acid.

Various *in vitro* biological activities were investigated. Nearly all the solvent extracts displayed anti-oxidant activity with IC_{50} values ranging from 1.61 to 74.50 µg/ml using the $DPPH^{\cdot}$ radical, while the IC_{50} values ranged from 11.88 to 69.26 µg/ml with the $ABTS^{\cdot+}$ radical. The solvent extract of *S. schlechteri* was three times more active than vitamin C. Total phenolic content based on gallic acid equivalents (GAE) revealed the presence of total soluble phenolics in the extract at 45 to 211 mg of GAE dry sample. Almost all the essential oils exhibited promising anti-inflammatory activity (5-lipoxygenase assay) with IC_{50} values ranging from 22.81 to 77.32 µg/ml. The antimalarial activity was determined using [^{3}H]-hypoxanthine method on the *Plasmodium falciparum* (FCR-3) strain. The IC_{50} values of the essential oils ranged from 1.20 to 13.50 µg/ml and were low compared to the solvent extracts (IC_{50} values ranging from 3.91 to 26.01 µg/ml). Betulafolientriol oxide and salvigenin isolated from *S. radula* inhibited the growth of malaria parasites with IC_{50} values of 4.95 and 24.60 µg/ml, respectively. With the exception of *S. radula*, all the solvent extracts displayed moderate to good activity against *Staphylococcus aureus,*

Bacillus cereus, Klebsiella pneumoniae, Escherichia coli and *Mycobacterium tuberculosis* with the MIC values ranging from 0.03 to 8.00 mg/ml. Four compounds, namely carnosol, 7-*O*-methylepirosmanol, oleanolic acid and its isomer ursolic acid were isolated from *S. chamelaeagnea* as the active principles against *S. aureus*. The solvent extracts of *Salvia* species were tested for *in vitro* anticancer activity against human breast adenocarcinoma (MCF-7), colon adenocarcinoma (HT-29) and glioblastoma (SF-268) using the sulforhodamine B assay. The extracts inhibited cell proliferation of all three cell lines to varying degrees, with the IC_{50} values ranging between 9.69 and 43.65 µg/ml and 8.72 and 59.12 µg/ml against the MCF-7 and SF-268 cell lines, respectively. The IC_{50} values against the HT-29 cell line ranged from 17.05 to 57.00 µg/ml. The *in vitro* toxicity profile of 28 samples (17 solvent extracts and 11 essential oils) was evaluated on human kidney epithelial cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5 dimethyl tetrazolium bromide method. The samples displayed some degree of toxicity with IC_{50} values ranging from 1.79 to 22.9 µg/ml for the essential oils and from 12.12 to 53.34 µg/ml for the solvent extracts. The essential oil composition of *S. africana-caerulea*, *S. africana-lutea* and *S. lanceolata,* collected at the same locality throughout the 2004/2005 growing season, was compared in terms of essential oil yields, chemical composition and biological activities. Mostly quantitative, rather than qualitative variation was observed. Major seasonal fluctuations of certain essential oil compounds were observed in all three species. Variations in biological activities of the solvent extracts over seasons were noted. The biological activities of the solvent extracts of three *Salvia* species (*Salvia africana-caerulea*, *S. africana-lutea* and *S. lanceolata*) were evaluated in the presence and absence of essential oils. The solvent extract of *S. africana-caerulea* without essential oil exhibited the best activity against Gram-positive bacteria (MIC value: 0.1 mg/ml), while the solvent extract containing essential oil of *S. africana-lutea* was the most active against Gram-negative bacteria. The toxicity profile of all three species was significantly higher ($P < 0.05$) with the solvent extracts containing essential oils. The *in vitro* biological activities add scientific support to the use of *Salvia* species in traditional medicine.

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Awards

Best poster presentation (3rd place) at the at the 30th Annual Congress of the South African Association of Botanists, University of KwaZulu-Natal, Durban, South Africa.

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%: Percent

° C: Degree Celsius

5´-FU: 5´-Fluorouracil

Abs: Absorbance

ABTS: 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid

$ABTS^{\cdot+}$: 2,2′-azino-bis- (3-ethylbenzothiazoline-6-sulfonic acid radical

AIDS: Acquired immuno deficiency syndrome

amu: Atomic mass units

ANOVA: One-way analysis of variance

ATCC: American type culture collection

br: Broadened

CCM: Complete culture medium

CD_3OD: Methanol

$CDCL_3$: Chloroform

CFU: Colony forming unit

CGT: Capitate glandular trichome

cm^2: square centimetre

CO_2: Carbon dioxide

COSY: Correlation spectroscopy

CPM: Counts per minute

CUT: Cuticle

d: Doublet

dd: Double of doublets

DEPT: Distortionless enhanced polarisation transfert

DMEM: Dulbecco's Modified Eagle's

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DPPH: 2,2-diphenyl-1-picrylhydrazyl

$DPPH^{\cdot}$: 2,2-diphenyl-1-picrylhydrazyl radical

EDTA: Ethylene diamine tetra-acetic acid

EI: Electron impact

Eq.: Equation

eV: Electron volt

FAA: Formaldehyde, alcohol and acetic acid

FBS: Fetal bovine serum

FCR: *Falciparum* chloroquine resistant

FCS: Fetal calf serum

FIC: Fractional inhibitory concentration

g: Gram

GAE: Gallic acid equivalent

GC: Gas chromatography

GC-MS: Gas chromatography coupled to mass spectrometry

GI: Growth index

GMA: Glycol methacrylate

h: Hour

H: proton

HEPES: [N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)]

HMBC: Heteronuclear multiple-bond correlation

HMQC: Heteronuclear multiple quantum

HPLC: High performance liquid chromatography

HT-29: Hypertriploid

Hz: Hertz

i.d.: Inner diameter

IC_{50}: Inhibitory concentration (concentration that reduces the effect by 50%)

INT: *p*-Iodonitrotetrazolium

***J*:** Coupling constant

kgf/cm^2: Kilogram force per square centimetre

kV: acceleration voltage

l: Litre

LM: Light microscopy

m/z: Mass to charge ratio

M: Metre

M: Molar

m: Multiplet

M^+: Molecular ion

max: Maximum

MCF-7: Michigan cancer foundation

mCi: Millicurie

mg: Milligram

MHz: Megahertz

MIC: minimum inhibitory concentration

min: Minute

ml: Millilitre

mm: Millimeter

mM: Millimolar

mm^2: Square millimetre

mmol: Milimole

MS: Mass spectrometry

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5 dimethyl tetrazolium bromide

***n*:** Number of repetitions

N_2: Nitrogen

nc: Not calculated

NCI: National cancer institute

nd: Not determined

NGT: Non-glandular trichome

NHLS: National health laboratory services

nm: Nanometer

NMR: Nuclear magnetic resonance

NOESY: Nuclear overhauser effect

NTCC: National type culture collection

O_2: Oxygen

PBS: Phosphate buffer saline

PDA: Photodiode array

PGT: Peltate glandular trichome

pH: Potential hydrogen

ppm: Parts per million

PRBC: Parasitized red blood cells

q: Quartet

***r*:** Pearson's correlation

***r^2*:** correlation coefficient

RBC: Red blood cell

R_f: Retention factor

RI: Retention indices

rpm: Revolution per minute

RPMI 1640: Roswell park memorial institute

Rt: Retention time

s.d.: Standard deviation of the mean

s.e.: Standard error of the estimate

s: Second

s: Singlet

S_{corr}: Correlation coefficient

SEM: Scanning electron microscopy

SRB: Sulforhodamine B

t: Triplet

TB: Tuberculosis

TCA: Trichloroacetic acid

td: triple of doublets

TLC: Thin layer chromatography

TMD: Thermabeam mass detector

TMS: Tetramethylsilane

TSA: Tryptone soya agar

TSB: Tryptone soya broth

U: Enzyme unit

u: Units

UI-A: Unidentified A

UI-B: Unidentified B

UI-C: Unidentified C

UI-D: Unidentified D

UV: Ultra violet

UV-VIS: Ultraviolet-visible

v/v: Volume per volume

w/v: Weight per volume

w/w: Weight per weight

WHO: World Health Organization

δ_c: Carbon shift

ΔGI: Difference in the growth index

δ_H: Proton shift

λ: Wavelength

µA: Microampere

µg: Microgram

µl: Microlitre

µm: Micrometre

µM: Micromolar

Chapter 1: General Introduction

1.1 Introduction

Traditional medicine is widely practiced in many Third World countries. The WHO estimated that about 80% of populations in developing countries rely on traditional medicine for their primary health care needs (Jäger and van Staden, 2000; Matu and van Staden, 2003). In rural areas, reliance on traditional medicine is high and can be attributed to both economic and cultural factors. From an economic point of view, the high cost of imported, conventional drugs and/or inaccessibility to western health care facilities implies that the traditional mode of health care is the only form that is affordable to them (Matu and van Staden, 2003). Even when western health care facilities are available, they usually exist side by side with traditional medicine (Sindiga, 1994). The use and preparation of medicinal plants is conveyed verbally from one generation to the next, a process that involves the risk of losing essential details (Atindehou *et al*., 2004). Furthermore, plant species that form the basis of traditional medicine are disappearing at an alarming rate in many countries of the world. It is therefore imperative to study medicinal plants and to determine and evaluate their potential biological activities and efficacy.

Secondary metabolites from a vast array of plants are used in numerous fields such as phytotherapy, perfumes, cosmetics, aromatherapy and spices. Aromatherapy has attracted the attention of many scientists and the screening of plants to study their biological activities has increased (Lahlou, 2004). For thousands of years, medicine and natural products have been closely linked with traditional medicines and chemical studies of these predominantly plant-derived traditional medicines were the basis of most early medicine such as aspirin and quinine (Butler, 2004). More recently, essential oils and various other extracts have stimulated a growing interest in natural product research and many have been screened for their potential use as alternative remedies for the treatment of various ailments (Buchbauer, 2000).

Species of the genus *Salvia* (Lamiaceae) are well known throughout the world for their medicinal properties which have made it an attractive choice for many researchers. Although some *Salvia* species have been scientifically studied in many parts of the world and are reported to have various biological activities including antibacterial (Ulubelen *et*

al., 2001), anti-oxidant (Couladis *et al*., 2003), anti-inflammatory (Perry *et al*., 2003, anticancer (Liu *et al*., 2000) and anticholinesterase (Perry *et al*., 2003), not much is known about the South African species. Therefore, there is little scientific basis to validate the claims by traditional medicine practitioners about the effectiveness of local indigenous *Salvia* species.

1.2 The role of secondary metabolites in plants

Plants are essential for all life on Earth since they provide oxygen. In addition, they provide non-nutritional compounds or secondary metabolites which are not necessarily involved in cell metabolism (e.g. essential oils). These natural compounds are formed from diverse precursors such as acetyl-CoA or amino acids, through enzymatic reactions (Harbone, 1993).

Plants are sessile organisms and can be attacked easily by snails, insects or vertebrate herbivores. Some authors have suggested that natural compounds might have evolved to provide protection to the plant against attack by herbivores and/or to challenge hostile environments. Experimental evidence supports the view that many alkaloids, cyanogenics, glycosides, glucosinolates, terpenoids and tannins are allochemicals (Wink, 2003). However, plants often need animals to assist in pollination or seed dispersal and in these cases, secondary metabolites serve to attract the animals to the plant. Some secondary metabolites concomitantly carry out physiological functions. For instance, peptides (protease inhibitors) can serve as mobile and toxic nitrogen transport (Harbone, 1993). The observed multiple functions are typical and do not contradict the main role of many secondary metabolites as chemical defense and signal compounds (Wink, 2003).

Secondary metabolites are present in all higher plants and display immense structural diversity (Table 1.1). As a rule, a single group of secondary metabolites dominate within a given taxon (Wink, 2003) although few major compounds are often accompanied by several derivatives and minor compounds. Typical secondary metabolites of the Lamiaceae include various terpenoids, especially monoterpenes, sesquiterpenes, as well as various phenolic acids (Wink, 2003). The secondary metabolites are generally concentrated in one particular region of a plant such as the leaves, roots, bark, fruits or glandular hairs. In cases where they occur in various organs of the same plant, they frequently have different chemical profiles (Araújo *et al*., 2003).

Table 1.1 Structural diversity and number of secondary plant metabolites (Wink, 2003).

Natural products	Number
Alkaloids[a]	12,000
Alkamides[a]	150
Amines[a]	100
Carbohydrates[b]	> 200
Cyanogenic glycosides[a]	60
Diterpenes[b]	2,500
Flavonoids[b]	4,000
Glucosinolates[a]	100
Monoterpenes[b]	2,500
Non-protein amino acids[a]	700
Phenylpropanoids, coumarins, lignans[b]	2,000
Polyacetylenes, fatty acids, waxes[b]	1,000
Polyketides[b]	750
Sesquiterpenes[b]	5,000
Tetraterpenes[b]	500
Triterpenes, saponins, steroids[b]	5,000

[a] with nitrogen [b] without nitrogen

The essential oils of plants are important not only because they possess biologically active compounds, but their chemical compositions have been used to resolve some taxonomic problems and discrepancies, especially in the Lamiaceae (Wink, 2003). It is thought that trichomes are structures responsible for the secretion of essential oils. These structures are also important in plant taxonomy, as they have been used to determine the degree of similarities among many species of the Lamiaceae. Considering the difficulties often encountered in distinguishing *Salvia* species, the study of their chemical composition and the morphology of the leaf trichome types can be an important tool in clarifying this issue.

1.3 Natural compounds and chemotherapeutic agents

Despite competition from synthetic drugs, natural compounds still occupy a commanding position among drugs that are now used against infective organisms and diseases. Table 1.2 lists a few isolated compounds from plants which are currently in clinical use. Drugs derived from natural compounds are well represented in the top 35 worldwide ethical drug sales between 2000 and 2002 with 40% being derived from natural compounds in the year 2000 (Butler, 2004).

Table 1.2 Natural compounds or naturally derived drugs from plants that are currently used clinically (Iwu, 1994; Butler, 2004).

Compounds / drugs	Plant	Disease
Artemisinin	*Atermisia annua*	Malaria
Aspirin	*Salix* species	Inflammation, pain
Physostigmine	*Physostigma venonosum*	Glaucoma
Quinine	*Cinchona pubescens*	Malaria
Reserpine	*Rauvolfia serpentia*	Hypertension
Taxol	*Taxus brevifolia*	Ovarian and breast cancer
Vinblastine and vincristine	*Catharanthus roseus*	Lung cancer

Natural compounds often have complex chemical structures making them difficult, if not impossible, to synthesize. Medicinal plants which form the backbone of traditional medicine have in the last few decades been subjected to intense pharmacological investigations. This has been catalysed by the acknowledgement of the value of medicinal plants as a potential source for new compounds with therapeutic value and as valuable sources for drug discoveries and development (Matu and van Staden, 2003). The common practice of phytochemists to isolate, characterize and publish phytocompounds without exploring the bioactivity is no longer attractive to academic journals and funding companies (Fatope *et al*., 2000). Therefore, it is important to isolate and identify the active compounds which show good biological activity from plants.

1.4 Approach to plant-based research

In the approach of searching for new drugs, no decision is taken by the researcher about the specific plant or organisms to be examined. Based, for example, on the impressive quantity and quality of information derived by indigenous cultures that have used these medicinal plants, plants are ethnobotanically selected with the aim of searching for compounds with desirable biological activities.

Drug discovery is often refered as a 'number-game'. There are an estimated 250,000 to plant species that inhabit our planet and only a very small percentage of these have been evaluated for potential bioactivities (Clark, 1996). Based on this approach, it would take many years of a systematic and concerted effort to evaluate the Earth's plants for a specific biological activity. Furthermore, it is a difficult task in terms of financial demands. Another problem that might arise from this approach is that extracts might contain more than one substance, which can obfuscate the assay. Results obtained from the early 1980's by random screening have been disappointing. Valeriote *et al*. (1990) estimated that only 1% of randomly selected agents tested for anticancer activity demonstrated sufficient solid tumour selectivity to proceed to *in vivo* experiments. Corbett *et al*. (1990) screened more than 15,000 agents against various cancer cell lines and found that only about 5% were selective *in vitro*. To date, this approach constitutes the only effective way to obtain new and unique classes of agents with novel mechanisms of action (Corbett *et al*., 1990). It is estimated that approximately 77% of the medicinal agents worldwide were obtained from ethnobotanical surveys (Corbett *et al*., 1990).

1.5 Future of natural products research

Although plants have, up to now, supplied man with a large number of excellent drugs and templates for further developments, some people have expressed concern feeling that research in this field is languishing. In the 1980's, there was a lack of emphasis on plant drug research in some developed countries because the available programmes did not successfully yield any new marketable medicines. After testing approximately 40,000 plants, the National Cancer Institute (USA) was unable to identify a single new agent to be used in the treatment of human cancer (Tyler *et al*., 1988). During the same period in Germany, drugs made from plants and even new plant constituents were continually

introduced into the market. The discovery of new drugs is a question of hard work and perseverance, as was shown by the National Cancer Institute when in the late 1980's it developed an exciting new drug (Taxol®) because of its continued efforts (Clark, 1996). Since the end of the twentieth century, there has been a resurgence of interest in the investigation of natural materials as sources of potential new chemotherapeutic agents (Cragg *et al*., 1997, Cragg and Newman, 2005). Research activity is stronger now than perhaps at any other time and this renewed interest can be attributed to several factors:

(i) unmet therapeutic needs,
(ii) remarkable diversity of both chemical structure and biological activity of naturally occurring secondary metabolites,
(iii) utility of novel bioactive natural products,
(iv) development of novel and sensitive techniques to detect biologically active natural products, and
(v) improved techniques to isolate, purify and structurally characterize these active constituents.

By coupling the technological capabilities with the creativity and innovation of nature, the future of natural product based research is more promising than ever before. Natural products are still a potential source of new drugs, especially in the anticancer and antihypertensive therapeutic areas. The natural products discovered to date have played a vital role in improving the human condition and this role will continue as long as there are unexplored sources of novel natural products (Clark, 1996).

1.6 The genus *Salvia*: description, history and traditional uses in South Africa

1.6.1 Morphological description

Salvia species are easily recognized by their square stems and opposite, simple pairs of leaves that are usually velvety or hairy. Leaves are often rugose, entire, toothed and lobed. Flowers are clustered in racemes, spike-like racemes, spikes and panicles are usually large and brightly coloured, depending on species. Flowers and stems are key diagnostic characteristics for identification of the genus (Hedge, 1974; Codd, 1985). There are four

stamens, but only two bear anthers. Seeds, stem cuttings and divisions are the common mode of propagation. *Salvia* grows optimally in full sun and needs well-drained soil. In waterlogged soil, the roots may rot.

1.6.2 History

Salvia species (sage) belong to the mint family Lamiaceae (formerly Labiatae). The genus name *Salvia* L. is derived from the Latin *salvare* meaning 'to heal or to be safe and unharmed' referring to the medicinal properties of some of the species (Blumenthal *et al*., 2000). It has been used for centuries, especially by the Chinese as a herb for longetivity or in Roman ceremonies as a sacred herb. This name was translated to *sauge* (sage) in French and *sawge* in Old English. Sage encompasses about 900 species, widespread throughout the world (Codd, 1985; Paton, 1991; Gali-Muhtasib *et al*., 2000) and includes several ornamental, culinary and medicinal species. Because they readily cross-pollinate, innumerable hybrids, both natural and manmade, are also found. The genus has a sub-cosmopolitan distribution, but is largely absent in the North and most of the low-lying tropical areas of the world such as the Amazon basin and central and west Africa (Paton, 1991). Most of South African species are found in the Cape region (Codd, 1985).

Although Mexico has the highest number of species (about 250), the center of origin of the genus has been reported to probably be Afghanistan and Soviet Central Asia where there are a larger range of primitive morphological types (Paton, 1991). In Africa, the greatest number of species is in the north-west and in the southern parts (Hedge, 1974). The genus is absent from most of western and central tropical Africa (Jäger and van Staden, 2000). The distribution of the genus extends all over northern Africa from west to east and southwards to the east African highlands. Although the species in the north-west are either common or affiliated to others species, most southern Africa species are endemic and only display a certain degree of similarity with other African species (Paton, 1991).

Southern africa is home to more than 24,000 higher plant taxa (Arnold and De Wet, 1993) and a large proportion of them are endemic in character (Mulholland and Drewes, 2004). In this part of the world, as in many Third World countries, traditional medicine still plays an important role with *Salvia* species being widely used alone or in combinations with other plants. About 26 species have been identified in South Africa (Codd, 1985; Paton,

1991) but species such as *S. coccinea*, *S. officinalis*, *S. reflexa*, *S. sclare, S. verbenaca* and *S. tillifolia* have been introduced and are thus not indigenous to the region.

1.6.3 Traditional uses of *Salvia* species

S. officinalis is native to the Mediterranean rim, especially around the Adriatic Sea and cultivated in South Africa and has one of the longest histories of use as a medicinal plant. *Salvia* has been used throughout the world and the famous proverb 'why should he die who has sage in his garden' was used to describe the medicinal properties of the plant (Gali-Muhtasib *et al*., 2000). A few centuries ago, it was nearly always at the top of the list of household remedies for the relief of many diseases. Herbalists in Jordan, Syria and Lebanon consider *Salvia* as a 'panacea' i.e. a universal drug (Gali-Muhtasib *et al*., 2000). The therapeutic value of *Salvia* was also recognized by the king of France, Louis XIV, who trusted *Salvia* more than his doctor (Gali-Muhtasib *et al*., 2000). The traditional uses of *Salvia* in South Africa are summarized in Table 1.3. Despite the fact that an important local ethnobotanical bibliographic description of the most commonly used plants in the treatment of various diseases exits (Watt and Breyer-Brandwijk, 1962; van Wyk *et al*., 2000), there are few experimental studies which validate the therapeutic properties of these plants locally.

1.7 Chemical constituents of *Salvia* species

1.7.1 Volatile compounds (essential oils)

Limited phytochemical work has been carried out on indigenous *Salvia* species. The essential oil of *S. stenophylla* collected on the "highveld" of the Free State revealed the presence of monoterpenes. The oxygenated monoterpenoids constituted 5% of the oil and the sesquiterpene hydrocarbons 35.5%. *S. stenophylla* was found to contain very low amounts of 1,8-cineole and camphor, and lacked α- and β-thujene (Jequier *et al*., 1980). Brunke and Hammerschmidt (1985) also analyzed the essential oil of *S. stenophylla* and reported the predominance of α-bisabolol (29.8%).

For the exotic *Salvia* species, the essential oil composition varies between species and 1,8-cineole, α-thujene, β-thujene, camphor, (*E*)-caryophyllene, linalool and linalyl acetate were

found to be the most predominant compounds in the majority of species investigated (Topcu *et al*., 1997; Gali-Muhtasib *et al*., 2000; Kalemba and Kunicka, 2003; Pitarokili *et al*., 2003; Pavela, 2004). Azcan *et al.* (2004) investigated the fatty acid seed oil composition of twelve *Salvia* species growing in Turkey and the main fatty acids found were oleic, linoleic, linolenic acids and palmitic acids.

Table 1.3 Medicinal uses of South African *Salvia* species (Watt and Breyer-Brandwijk, 1962).

Species	Part used	Mode of uses	Ailments
S. africana-caerulea	Twig	Infusion mixed with Epson salt and lemon	Colic, diarrhoea, indigestion, abdominal trouble
S. africana-lutea	Whole plant	Infusion, decoction	Colds, cough
S. chamelaeagnea	Leaves	Decoction, infusion	Colds, cough
S. triangularis	Whole plant	Decoction with *Helichrysum latifolium* and *Commelina africana*	Liver sickness, barrenness
S. repens	Roots, leaves, whole plant	Decoction, added to bath	Sores on the body, stomach problems, diarrhoea
S. runcinata	Whole plant, leaves, stems and roots	Burned, decoction	Disinfect dwelling sickness, urticaria
S. scabra	Leaf	Decoction	Purgative
Salvia species	Roots, whole plants	Decoction	Biliousness, cold, febrile attacks
Salvia species	Whole plant	Infusion in combination with *Leonotis leonurus*	Tuberculosis, cough, influenza, bacterial infections, cold
S. stenophylla	Whole plant	Infusion, decoction	Disinfect dwelling sickness

1.7.2 Non-volatile compounds (phenolic compounds)

Salvia species are a rich source of polyphenolics and flavonoids. The polar phenolic acids constitute the major part of the *Salvia* decoction. Caffeic acid plays a central role in the biochemistry of Lamiaceae and occurs predominantly in the dimer form as rosmarinic acid (Gerhardt and Schroeter, 1983). In *Salvia* species, caffeic acid is the building block of a variety of the plant metabolites from the simple monomers to multiple condensation products that give rise to a variety of oligomers (Lu and Foo, 2002). Phenolic acids such as rosmarinic, caffeic and salvimanolic acids have been isolated (Lu and Foo, 2002).

The phenolic glycosides are not very common in *Salvia*. The rosmarinic acid 3'-glucoside and its methyl ester, as well as the *cis*- and *trans*-*p*-coumaric acid 4-*O*-(2-*O*-apyiosyl) glucosides, are the only examples of the glycosylated phenolic acids (Lu and Foo, 2002). In contrast, flavonoids are widely distributed in *Salvia* species (Ulubelen and Tuzlaci, 1990) and they are mostly present as flavones, flavonols and their glycosides (Lu and Foo, 2002). The majority of *O*-flavonoids are the flavones, apigenin and luteolin. The 6-hydroxyflavones are the flavonoids that characterise the species of *Salvia* and they include a variety of 6-hydroxylated apigenin and luteolin derivatives with cirsimaritin. Flavonols are mostly those of kaempferol and quercetin. Flavonone *O*-glycosides are apparently common in *Salvia* and most of them are flavone 7-glucosides represented by cosmosiin and cinaroside (Lu and Foo, 2002).

A solvent extract of *S. stenophylla* collected in the Eastern Cape was investigated for flavonoids. Apigenin-7-methyl ether, luteolin and 6-hydroxyluteolin-6,7-dimethyl ether were identified (Wollenweber *et al*., 1992). A large study on the occurrence of alkaloids in plants included a number of the southern African *Salvia* species, with *S. chamelaeagnea*, *S. namaensis* and *S. runcinata* testing positive for alkaloids, while in *S. africana-caerulea*, *S. africana-lutea*, *S. coccinea*, *S. dolomitica*, *S. reflexa*, *S. tillifolia* and *S. triangularis*, no alkaloids were detected (Raffauf, 1996).

1.8 Biological activities of *Salvia* species

A literature search indicated that the genus *Salvia* has been a popular topic in phytochemical and ethnobotanical research. The solvent extracts, essential oils and compounds isolated from *Salvia* species revealed that they displayed a broad range of pharmacological properties, both *in vitro* and *in vivo*. Table 1.4 lists some examples of biological properties of *Salvia* including two indigenous species.

1.9 Rationale and motivation of the study

Salvia was chosen as the basis of this study because of its long history of traditional use in South Africa and the rest of the world. Despite many reports on the medicinal properties of *Salvia*, few studies have been carried out on indigenous species (Table 1.4).

Table 1.4 Some examples of biological activities of *Salvia* species.

Species	Plant parts used	Bioactive fraction/compound	Activity	Methodology used/ organisms used
S. aethiopis	Roots	Aethiopinone	Anti-inflammatory	5-Lipoxygenase [a, b]
S. africana-lutea	Leaves	Solvent extract	Anti-analgesic	Acetic acid writhing in mice [c]
S. fruticosa	Whole plant	Rosmarinic acid	Anti-oxidant	Free radical assay [d]
S. hypargeia	Roots	Crude extracts, taxodione	Antibacterial	*Mycobacterium tuberculosis* [e]
S. lavandulaefolia	Aerial parts	Essential oils	Anticholinesterase	Microtitre plate assay [f]
S. miltiorrhiza	Roots	Tanshionones	Anticancer	Human tumour cells [g, h]
S. officinalis	Leaves	Ursolic acid	Anti-inflammatory	Oedema induced in mice [i]
S. paleastina	Leaves	Cirsimaritin	Antibacterial	Gram-positive bacteria [j]
S. prionitis	Leaves	3-Keto-4-hydroxysa-prothorquinone	Anticancer	Stomach cancer cell lines [k]
S. repens	Whole plant	Dichloromethane:methanol extract	Antimalarial	Parasite lactate dehydrogenase [l]
S. viridis	Roots	1-Oxoferrunol	Antibacterial	*S. aureus, B. cereus, B. subtilis* [m]

[a] Hernandez-Perez *et al*., 1995; [b] Benrezzouk *et al*., 2001; [c] Amabeoku *et al*., 2001; [d] Exarchou *et al*., 2002; [e] Ulubelen *et al*., 1988; [f] Savelev *et al*., 2003; [g] Liu *et al*., 2000; [h] Ryu *et al*., 1997; [i] Baricevic *et al*., 2001; [j] Miski *et al*., 1983; [k] Chen *et al*., 2002; [l] Clarkson *et al*., 2004; [m] Ulubelen *et al*., 2000.

From a literature survey on biological activities of the South African *Salvia* species, only two publications reported the pharmacological properties in *S. africana-lutea* and *S. repens* (Amabeoku *et al*., 2001; Clarkson *et al*., 2004) in addition to the composition of *S. stenophylla* essential oil (Jequier *et al*., 1980; Brunke and Hammerschmidt, 1985).

The reported traditional medicinal uses and the lack of phytochemical and pharmacological studies on indigenous *Salvia* species prompted this investigation into the phytochemistry and the biological activities of various *Salvia* species from South Africa.

1.10 Aim of the study

The aim of this study was to screen seventeen South African *Salvia* species in order to evaluate their biological activity, to isolate and identify compounds responsible for the selected biological activities and to assess a chemotaxonomic relationships amongst South African representatives of the genus *Salvia*.

1.11 Objectives of the study

The objectives of this study were to:

(i) examine the morphology of leaf trichome types of *Salvia* species and to assess the taxonomic value of these epidermal structures,

(ii) investigate the chemical composition of the essential oils and to assess variation in their chemical composition across the seasons using gas chromatography (GC) and gas chromatography coupled to mass spectrometry (GC-MS) analysis and to identify various groups that may be used in the flavourant and cosmetic industries,

(iii) identify the presence of phenolic compounds previously identified in exotic *Salvia* in the solvent extract of indigenous species using high performance liquid chromatography,

(iv) investigate the anti-oxidant activity of the crude extracts and essential oils of indigenous *Salvia* species using two radicals,

(v) evaluate the ability of crude extracts and essential oils to inhibit the 5-lipoxygenase enzyme which is one of the enzymes involved in the inflammatory process,

(vi) investigate the effect of the essential oils and solvent extracts on *Plasmodium falciparum*, the protozoan responsible for malaria by the [^{3}H]-hypoxanthine radiometric assay and isolate the active compound(s) from the most active solvent plant,

(vii) evaluate the inhibitory effects of the essential oils and solvent extracts on the growth of selected bacteria and the common tuberculosis pathogen as well as to isolate the active compound(s) from the plant showing the best antibacterial activity,

(viii) evaluate the inhibitory effects of the solvent extracts against the growth of breast, neuronal and colon cancer cell lines using a protein detection colorimetric method,

(ix) determine the toxicity profile of the essential oils and solvent extracts against a transformed human kidney epithelium cell line using the tetrazolium cell proliferative method,

(x) study the seasonal variation in biological activities of selected *Salvia* species, and

(xi) evaluate the influence of the essential oils on the biological activities of solvent extracts.

1.12 Outline of the thesis

The rest of the thesis consists of 13 Chapters. Reprints of publications, abstracts for conferences and the monographs are included in the Appendices. Apart from Chapters 2 and 14 which describe the localities and the general conclusions, respectively, each Chapter consists of an abstract, an introduction, materials and methods, results, discussion and conclusion.

Chapter 2: The extraction procedures for volatile (essential oils) and non-volatile (solvent extracts) fractions are described. The localities where the plants were collected are also listed.

Chapter 3: The variety of trichomes found in various plant organs have been a valuable tool for comparative systematic studies of Angiosperms. In this Chapter, the morphology of the leaf trichome types is examined.

Chapter 4: The essential oils have a broad range of applications, including the cosmetic industry, food industry and in aromatherapy. GC and GC-MS were used to analyse the chemical composition of the essential oils isolated by hydrodistillation. The relationship among different species could thus be more clearly established.

Chapter 5: *Salvia* is known to possess various phenolic compounds which contribute to biological activities. Some of the most common compounds in *Salvia* extracts as well as those isolated from two *Salvia* species are identified in the investigated species.

Chapter 6: There is increasing evidence to suggest that many degenerative diseases such as inflammation, brain dysfunction, immune system suppression and cancer could be the result of cellular damage caused by free radicals. The free radical scavenging activity (anti-oxidant activity) of the solvent extracts and essential oils is evaluated and the phenolic content determined.

Chapter 7: The 5-lipoxygenase enzyme is one of the key enzymes involved in the inflammation process. The potential of the solvent extracts and essential oils to inhibit this enzyme is evaluated.

Chapter 8: The increasing prevalence of malaria has been attributed to a number of factors, one of them being the emergence and spread of drug resistant parasites. In this Chapter, the essential oils and solvent extracts were tested for their potential to inhibit the growth of *Plasmodium falciparum* and two compounds from the most active solvent extract were isolated, characterised and tested.

Chapter 9: Despite the progress in understanding the life cycle and control of many pathogens, nearly all diseases, affecting millions of people in developing countries, are still caused by micro-organisms. The essential oils and solvent extracts were investigated in order to evaluate their potential to inhibit the growth of common bacterial pathogens. Furthermore, the active compounds were isolated and characterised from the most promising plant.

Chapter 10: A major problem with present day cancer chemotherapy is the serious deficiency of active drugs for the curative therapy and their adverse effects on the patient. The anticancer activity of solvent extracts is investigated on three different cell lines and their potential selectivity noted.

Chapter 11: There is a common notion among people who frequently use *Salvia* species to treat different ailments that 'if it does not benefit, it would not harm' and many people are not aware of any side effects or toxic symptoms that may be associated with excessive use of *Salvia* species. In this Chapter, the toxicity of essential oils and the solvent extracts is explored.

Chapter 12: The composition of the essential oils and solvent extracts may vary depending on environmental conditions and season which may, inevitably, affect the biological activities. Seasonal variation in essential oil composition and biological activities of the solvent extracts across seasons are evaluated for three species.

Chapter 13: Aromatic plants such as *Salvia* species possess both the volatile and non-volatile fractions. The contribution of the volatile fractions on the biological activities of the non-volatile fraction is discussed.

Chapter 14: General conclusions of the study and recommendations are presented. The reference documents employed for this research are cited subsequent to Chapter 14.

Chapter 2: Plant Collection, Isolation of Volatiles and Extraction of Non-Volatiles

2.1 Species selection

Species investigated in this study were selected according to their reported traditional uses in South Africa.

2.2 Collection of plant materials

Codd (1985) described 26 *Salvia* species in southern Africa, 17 of 22 species occurring naturally in southern Africa were included in this study. The aerial parts of the selected species were harvested at different localities in South Africa between December 2003 and December 2005. Possible variation between populations was not taken into account and only one representative per species was considered. The taxonomic identification of all species collected was confirmed at the herbarium at the South African National Biodiversity Institute (Pretoria) and voucher specimens were deposited in the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa. Table 2.1 lists the species investigated and a concise description and photographs of each species are shown in the monographs in Appendix C. The traditional uses of each plant are also included in Appendix C.

2.3 Preparation of samples

2.3.1 Preparation of the essential oils (volatile fractions)

The essential oils of the fresh aerial parts were isolated by hydrodistillation using a Clevenger type apparatus for three hours at 100 °C (Figure 2.1). The oil yield was recorded before being sealed in glass vials with silica septa, protected from light and kept refrigerated until used.

Table 2.1 Species studied, localities of collection, accession and voucher numbers.

Species	Locality	Date of collection	Accession number (KBG[1])	Voucher number
S. africana-caerulea	SWC[2]	01-12-2003	0554/82	AV 875
S. africana-lutea	SWC[2]	01-12-2003	1185/71	AV 873
S. albicaulis	KBG[1]	06-01-2004	0050/79	AV 894
S. aurita	KBG[1]	01-12-2003	0244/01	AV 1066
S. chamelaeagnea	KBG[1]	06-01-2004	0725/93	AV 848
S. disermas	KBG[1]	31-03-2005	0574/74	AV 1194
S. dolomitica	ex Manning	26-09-2003	0038/79	AV 838
S. garipensis	KBG[1]	27-03-2004	0689/91	AV 1193
S. lanceolata	SWC[2]	01-12-2003	0919/96	AV 877
S. muirii	KBG[1]	27-03-2004	189/82	AV 874
*S. namaensis**	Swartberg	11-07-2002	n.g[3]	AV 497
S. radula	Road to Derby	13-12-2003	n.g[3]	AV 880
S. repens	KBG[1]	06-01-2004	0609/95	AV 615
S. runcinata	Klerkskraal Dam	13-12-2003	n.g[3]	AV 679
S. schlechteri	KBG[1]	27-03-2004	0173/02	AV 1068
S. stenophylla	East of Clarens	06-01-2004	n.g[3]	AV 893
S. verbenaca	De Rust	02-12-2003	n.g[3]	AV 631

[1] KBG: Kirstenbosch Botanical Garden

[2] SWC: South Western Cape

[3] n.g: Accession number not given because the plants were not collected at the KBG

* Plant material used was collected in 2002

2.3.2 Preparation of the solvent extracts (non-volatile fractions)

Aerial parts of each plant were air dried at room temperature for seven days and pulverised into a fine powder, using a coffee grinder. The pulverised aerial parts were then extracted

with methanol:chloroform (1:1, v/v) in a water bath for three hours at 36-40 °C and filtered using Whatman® No 1 filter paper (pore size: 20-25 μm).

The filtrate was evaporated to dryness under reduced pressure and kept refrigerated until used. It is important to note that various solvent systems (acetone, methanol, dichloromethane, chloroform) were assayed before the methanol:chloroform mixture was finally selected because this mixture extracted compounds over a range of polarities.

Figure 2.1 Clevenger apparatus used for the isolation of the essential oils.

Chapter 3: Leaf Trichome Types

Abstract

Trichomes are outgrowths of the epidermis found on various plant organs. Leaf trichome morphology is described here for the first time for 12 species of *Salvia* collected at the Kirstenbosch Botanical Garden. Leaf surfaces were examined by scanning electron microscopy while transverse sections of leaves were studied by light microscopy. All species investigated contain both glandular and non-glandular trichomes. The non-glandular trichomes are multicellular, unbranched and consist mainly of elongated cells, while the glandular trichomes are either capitate or peltate. The capitate type, present in all species, has a unicellular or multicellular head, one to four short or long stalk cells and one epidermal basal cell. The peltate type, also found in all species, is relatively large with multicellular head cells (up to sixteen), one or two stalk cell(s) and one epidermal basal cell. The non-glandular hairs are abundant in *S. africana-lutea*, *S. lanceolata* and *S. muirii,* while many glandular trichomes are observed in *S. africana-caerulea*, *S. chamelaeagnea*, *S. disermas*, *S. garipensis* and *S. repens*. Few glandular trichomes were found in *S. albicaulis*, *S. dolomitica, S. lanceolata* and *S. muirii*. The non-glandular trichomes as well as the types of glandular trichomes observed are taxonomically informative to distinguishing between groups of species.

3.1 Introduction

Trichomes are hair-like outgrowths derived from specialised epidermal cells on leaf, root or stem surfaces. Scientific interest in plant trichomes is based on their taxonomic importance and on the economic benefit of some trichome-generated products (Davis and Heywood, 1973; Theobald *et al*., 1979).

The great diversity of trichomes and the composition of exudates produced by them are used in plant taxonomy to distinguish between closely related species in some families (Spring, 2000). There is a great range in coverage for both glandular and non-glandular trichomes, and various proportions of types between species, and between the abaxial and adaxial sides of organs such as leaves. Hairs may be present on one side or both sides equally or unequally, sometimes along the margins and/or veins or restricted to intercostal regions in clefts (Werker, 2000). When non-glandular trichomes form a dense indumentum, they may serve as a mechanical barrier against various external factors such as excessive water loss, pathogens and herbivores (Werker, 2000).

Although the role of glandular trichomes is not clearly elucidated, they are considered as storage sites of secondary metabolites. Glandular trichomes which secrete lipophilic substances may provide chemical or physiochemical protection against various types of herbivores and pathogens by entrapping, deterring, poisoning them or attract them for pollination or seed dispersal (Wagner, 1991).

Glandular trichomes vary in the chemical composition of substances they secrete, mode of production, location and function (Hanlidou *et al*., 1991; Ascensâo *et al*., 1995). Secretions include sugars, salts, lipids, essential oils, resins and proteins. Suggestions have been made to classify glandular trichomes according to the nature of their secretion products (Fahn, 2001). However, there are some limitations to this type of classification since some glandular hairs may secrete more than one type of substance (Werker, 2000).

The study of trichomes is a valuable tool in the resolution of many taxonomic problems in the Lamiaceae and may be used to determine the relationship between species (Davis and Heywood, 1973; Theobald *et al*., 1979). Although the indumentum is characteristic in many members of the Lamiaceae, little is known on the structural diversity of the

trichomes of South African *Salvia* species which might facilitate accurate identification. *Salvia* species are frequently used in traditional medicine and their scarcity makes them highly susceptible to adulteration and substitution. Thus, the wrong plant could easily be used in many herbal preparations with potentially dangerous consequences.

The objectives of this chapter were to:

(i) investigate the structure of trichomes using scanning electron microscopy (SEM) and light microscopy (LM) in order to determine the leaf trichome types in twelve South African species of *Salvia*, and

(ii) evaluate the structural characteristics of leaf trichomes for their potential in identifying species.

3.2 Materials and methods

3.2.1 Plant material

Fresh leaves of all 12 species, namely; *S. africana-caerulea, S. africana-lutea, S. albicaulis, S. aurita, S. chamelaeagnea, S. disermas, S. dolomitica, S. garipensis, S. lanceolata, S. muirii, S. repens* and *S. schlechteri*, were collected at the Kirstenbosch Botanical Garden (South Africa) in August 2005. For each species, some leaves were air-dried and others directly preserved in a formaldehyde, alcohol and acetic acid solution (FAA) basically according to Johansen (1940), but the proportions were modified as follows; distilled water:95% ethanol:formaldehyde:glacial acetic acid (28:17:6:2). Only one collection per species was considered.

3.2.2 Preparation of solutions and stain

3.2.2.1 Preparation of glycol methacrylate

The glycol methacrylate (GMA) monomer mixture was prepared using 188 ml of glycol methacrylate, 12 ml of polyethylene glycol 200 and 1 g of benzoyl peroxide. The mixture was stirred slowly for one hour and the solution filtered with Whatman® No 1 filter paper. The filtrate was placed in a desiccator and the air removed with a vacuum pump before being stored in the dark at 4 °C.

3.2.2.2 Preparation of toluidine blue

The buffer used to prepare the 0.05% (w/v) toluidine blue was made with 0.25 g benzoic acid and 0.3 g sodium benzoate dissolved by heating in 200 ml of distilled water. The pH was adjusted to 4.4 with NaOH and the solution was filtered through Whatman® No 1 filter paper and stored in the dark at room temperature.

3.2.3 Morphological and anatomical studies of leaf trichomes

3.2.3.1 Morphology of leaf trichomes: scanning electron microscopy

Leaves preserved in FAA were cut with a razor blade and rinsed with 50% ethanol five times for 30 minutes. The leaf was then dehydrated successively once in 70%, once in 90% and three times in 100% ethanol for 30 minutes followed by critical point drying in liquid carbon dioxide. For dried material, small pieces were obtained. Each leaf portion was then mounted on an aluminium stub by securing it with double-sided adhesive tape, coated with gold and examined. A standard location on each leaf (Figure 3.1) was chosen so that similar positions were examined. Each sample was scanned at a low magnification to reveal the general surface morphology and at higher magnification (where necessary) to show details. Both the abaxial and adaxial surfaces were studied. Samples were examined with a JEOL-5600 scanning electron microscope operating at an acceleration voltage of 6 kV. The trichome types were scored as absent (–) or present (+) and the relative abundance of glandular and non-glandular trichomes noted.

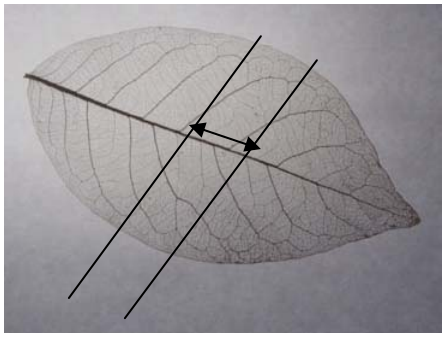


Figure 3.1 Leaf area selected for the study of trichome morphology.

3.2.3.2 Microscopic structure of trichomes: light microscopy

Small portions (≈ 3 mm^2) of leaves preserved in FAA were cut with a razor blade and prepared according to the method of Feder and O'Brien (1968). The material was

dehydrated by passing it through a graded alcohol series (2 x 50% ethanol, 2 x 100% ethanol; 2 x 100% propanol and 2 x 100% butanol). Specimens were left in each alcohol concentration for six hours to ensure that alcohol penetration was complete after which the material was infiltrated with the GMA monomer mixture. Each piece was then placed in a gelatin capsule containing GMA and incubated at 60 °C for 24 hours to polymerise prior to orienting and sectioning. Several transverse sections (3-5 μm thick) were cut using a Porter Blüm ultramicrotome and placed on slides with a drop of distilled water. Slides were placed on a slide warmer for the sections to adhere and then stained with undiluted Schiff's reagent and toluidine blue (as described in Section 3.2.2.2) (Feder and O'Brien, 1968). The slides were finally mounted with Entallen® and observations were made on a light microscope (Leitz Wetzlar) equipped with a digital camera (KY-F1030, JVC) connected to a computer with the Acquis Digital Imaging syncroscopy version 4.0.0.7. The relative abundance of trichomes was determined by counting the number of trichomes under the light microscope. At least 10 fileds were counted to obtain an average number.

3.3 Results

3.3.1 Scanning electron microscopy observations

Representative portions of leaf surfaces are shown in Figures 3.2 to 3.7. Two glandular trichome types, namely peltate and capitate could be observed and are present in all the investigated species. There is no difference in trichome type, between adaxial and abaxial surfaces in all taxa. Although the abundance of trichomes differs among the species with either glandular or non-glandular trichomes predominating. In order words, where the glandular trichomes are less dense, the non-glandular trichomes form a dense covering that completely obscures the epidermal surface and this was particularly evident in *S. africana-lutea* (Figure 3.2C), *S. dolomitica* (Figure 3.5A), *S. lanceolata* (Figure 3.6A) and *S. muirii* (Figure 3.6D). The non-glandular hairs are multicellular, unbranched and consist of long cells and are also present in all the species (Figures 3.2 to 3.7). While most non-glandular trichomes bear one basal cell, *S. aurita* (Figure 3.3D), *S. chamelaeagnea* (Figure 3.4A) and *S. repens* (Figure 3.7A) bore multiple cells at the base. Micropapillae were visible on the non-glandular trichomes of some species such as *S. chamelaeagnea* (Figure 3.4C), *S. lanceolata* (Figure 3.6C) and *S. muirii* (Figure 3.6E). In some species such as *S. africana-lutea* (Figure 3.2D), *S. albicaulis* (Figure 3.3B), *S. aurita* (Figure 3.3F) and *S.*

chamelaeagnea (Figure 3.4B), the rupture of the cuticle revealed that there are up to sixteen secretory cells present in the peltate trichomes, whereas the capitate trichomes are composed of up to four head cells and this can clearly be seen in *S. garipensis* (Figure 3.5F) and *S. muirii* (Figure 3.6E).

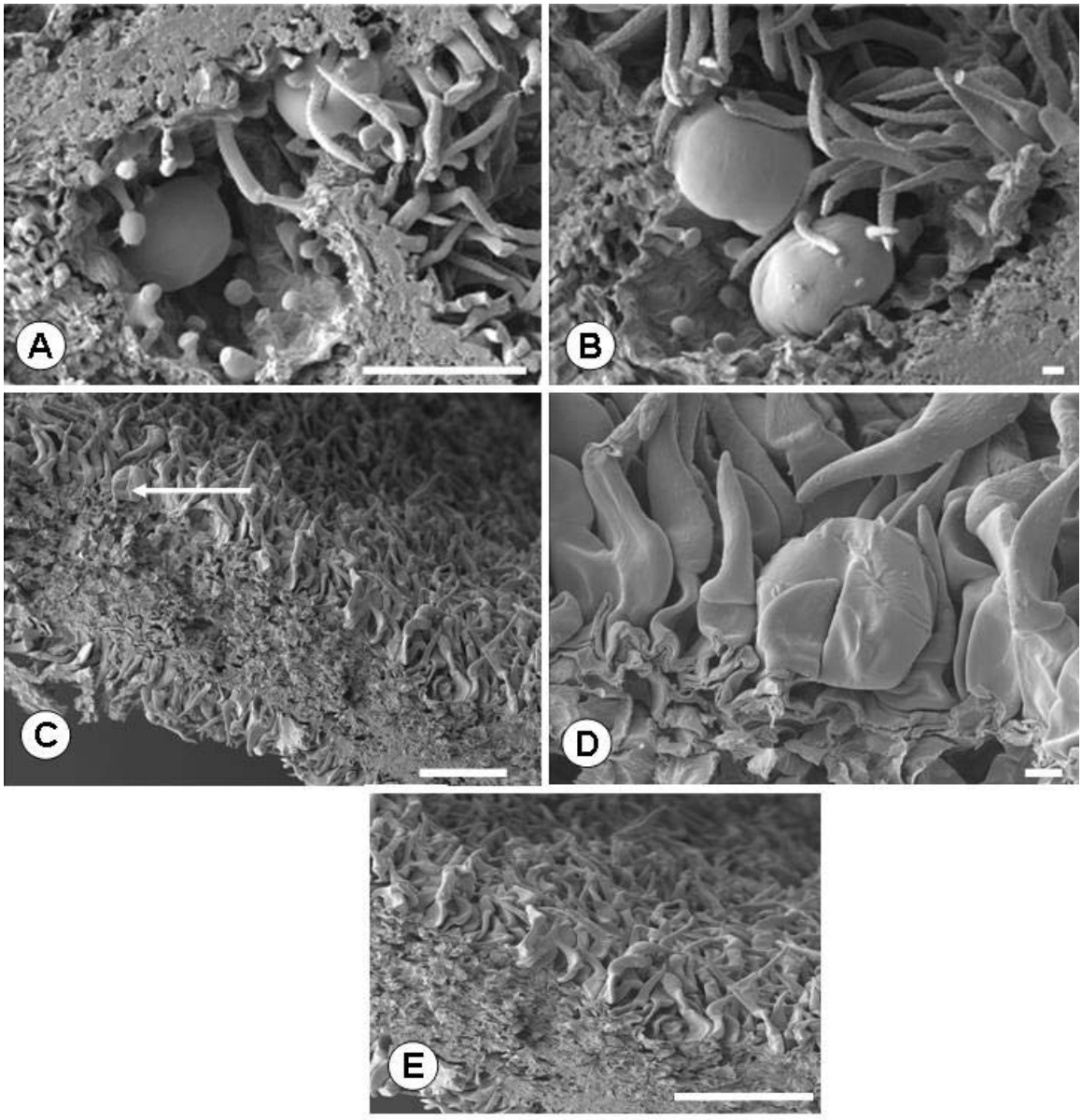


Figure 3.2 SEM micrographs of *Salvia africana-caerulea* (**A** and **B**) and *S. africana-lutea* (**C**, **D** and **E**). **(A):** Stalked capitate trichomes, sessile peltate trichomes and non-glandular trichomes on the lower leaf surface, bar = 100 µm; **(B):** sessile peltate trichomes on lower leaf surface covered by turgid and intact cuticle and non-glandular multicellular trichomes unbranched, bar = 10 µm; **(C):** leaf surfaces showing a glandular trichome (arrow) and dense covering of non-glandular trichomes, bar = 100 µm; **(D):** details of the lower leaf surface showing sessile glandular trichome and non-glandular trichomes, bar = 100 µm; **(E):** high density of non-glandular trichomes on the lower leaf surface, bar = 100 µm.

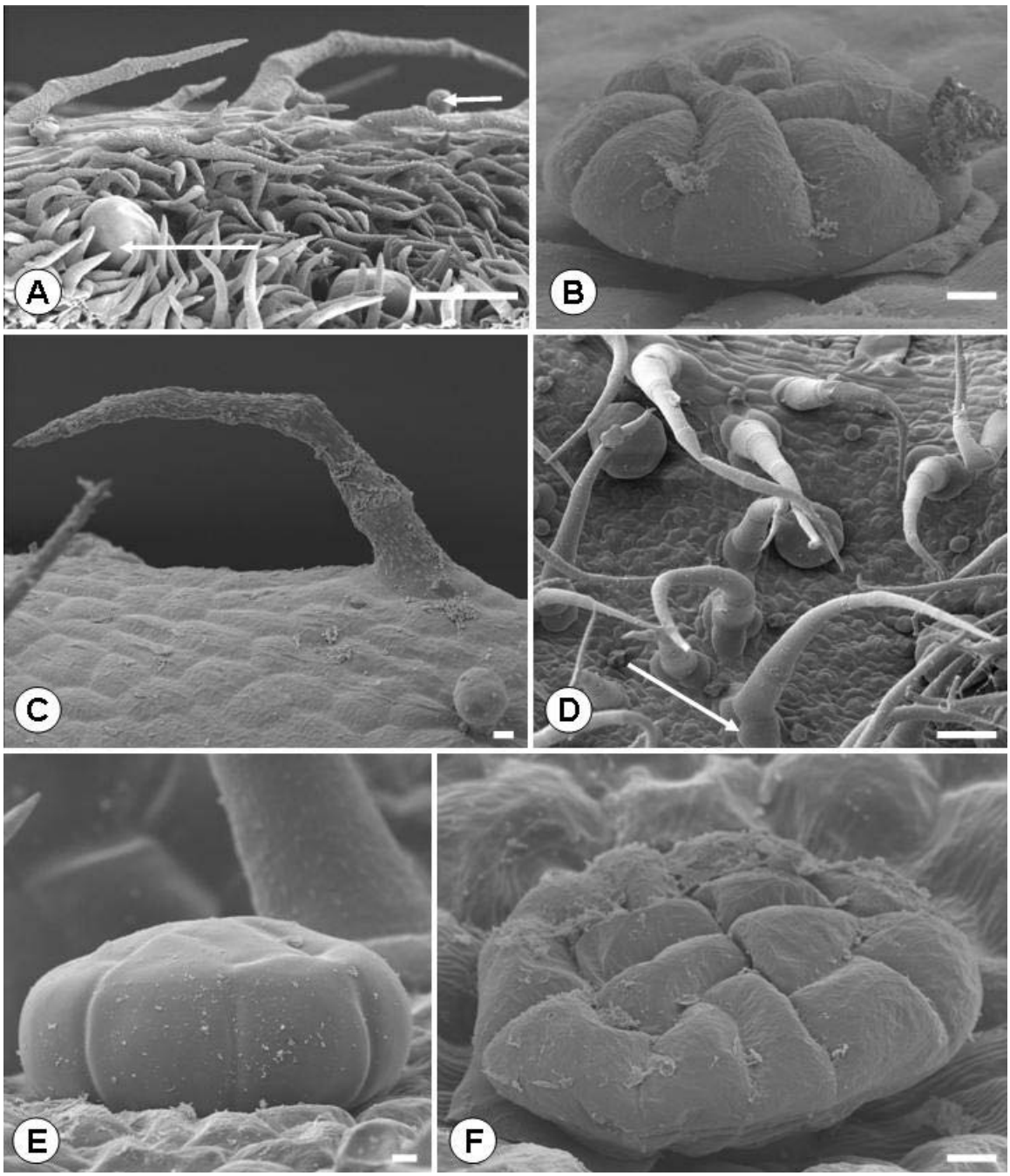


Figure 3.3 SEM micrographs of *Salvia albicaulis* (**A, B** and **C**) and *S. aurita* (**D, E** and **F**). **(A):** lower leaf surface showing a capitate (short arrow) and peltate (long arrow) trichomes, bar = 100 µm; **(B):** sessile peltate trichome on lower surface with a ruptured cuticle revealing individual secretory cells, bar = 10 µm; **(C):** non-glandular unbranched trichome on the upper leaf surface, bar = 10 µm; **(D):** peltate and capitate trichomes, the arrow indicates the base of a non-glandular trichome with multiple cells, bar = 100 µm; **(E):** peltate trichome on lower surface with intact cuticle, bar = 100 µm; **(F):** a multicellular peltate trichome with a ruptured cuticle, bar = 100 µm.

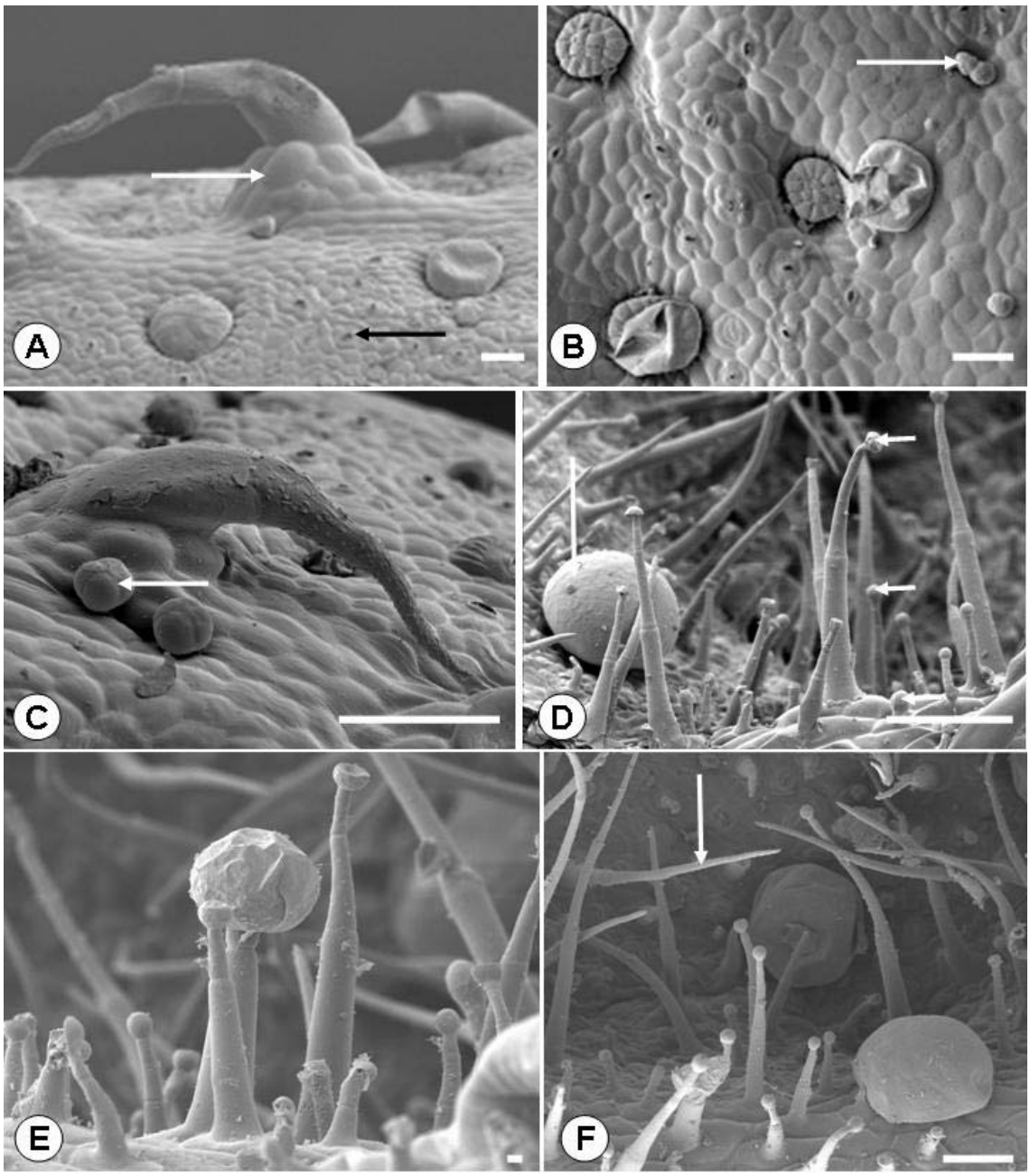


Figure 3.4 SEM micrographs of *Salvia chamelaeagnea* (**A, B** and **C**) and *S. disermas* (**D, E** and **F**). **(A):** lower leaf surface showing glandular trichomes; the black arrow indicates a stoma and the white arrow the base of a non-glandular trichome with many cells, bar = 100 µm; **(B):** sessile peltate trichomes on the lower surface with a ruptured cuticle revealing individual secretory cells and neigbbouring stomata; the arrow indicates a capitate trichome of two cells, bar = 100 µm; **(C):** non-glandular trichome, the white arrow indicates a capitate trichome composed of four cells, bar = 100 µm; **(D):** upper surface showing peltate (long arrow) and capitate (short arrows) trichomes, bar = 100 µm; **(E):** capitate trichomes with long and short multicellular stalk cells, rupture of the cuticle visible, bar = 100 µm; **(F):** capitate, non-glandular (arrow) and peltate trichomes with intact cuticles, bar = 100 µm.

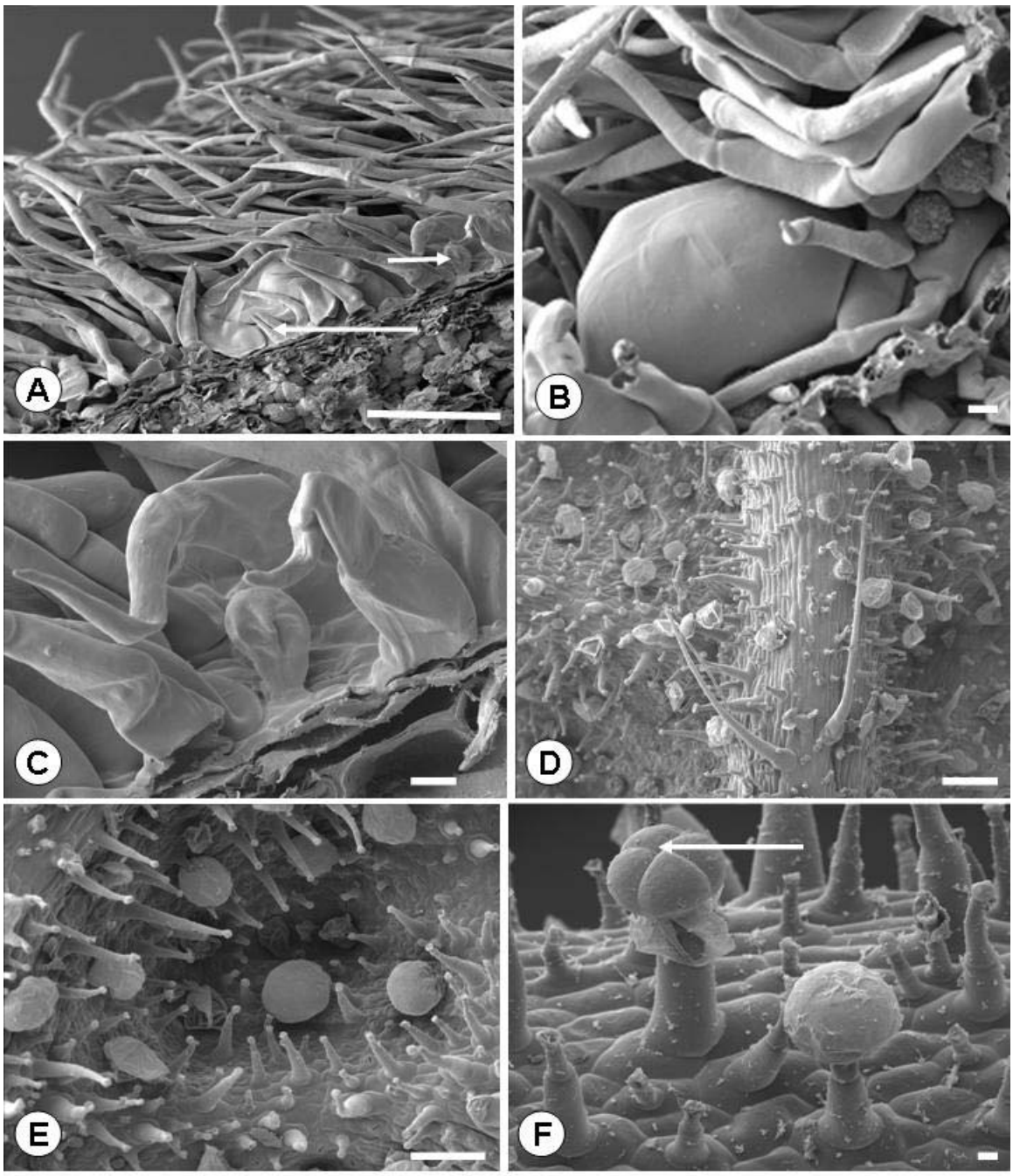


Figure 3.5 SEM micrographs of *Salvia dolomitica* (**A, B** and **C**) and *S. garipensis* (**D, E** and **F**). **(A):** upper leaf surface showing a peltate trichome (long arrow), a capitate trichome (short arrow) and a dense covering of non-glandular trichomes, bar = 100 μm; **(B):** detail of upper surface of sessile peltate and non-glandular trichomes, bar = 10 μm; **(C):** a capitate trichome with a short stalk, bar = 10 μm; **(D):** lower surface showing relatively long multicellular non-glandular trichomes (mainly on the vein), peltate and capitate trichomes with short and long multicellular stalk cells, bar = 100 μm; **(E):** numerous peltate and capitate trichomes, bar = 10 μm; **(F):** capitate trichomes, arrow indicates the four cells revealed by the ruptured cuticle, bar = 100 μm.

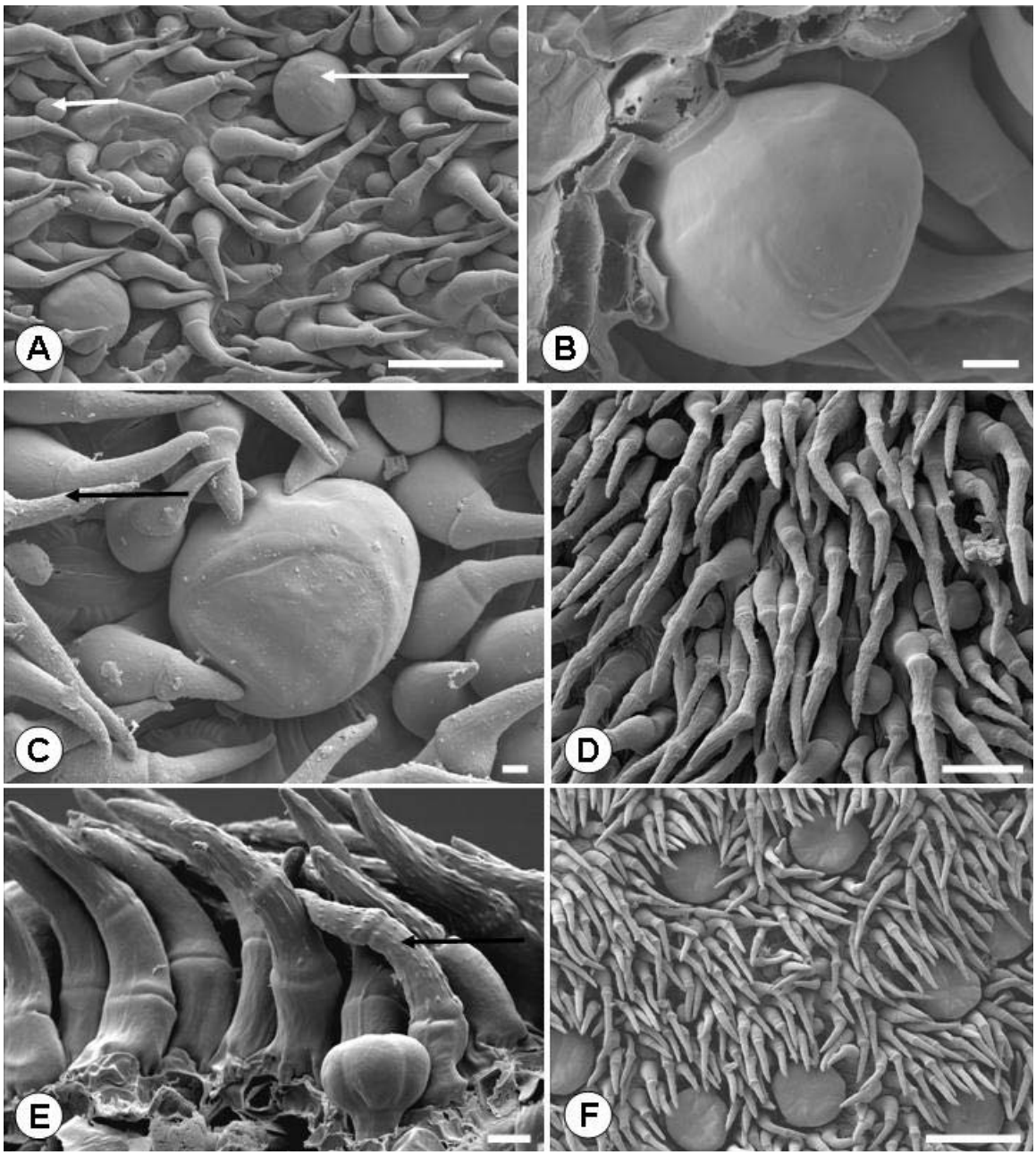
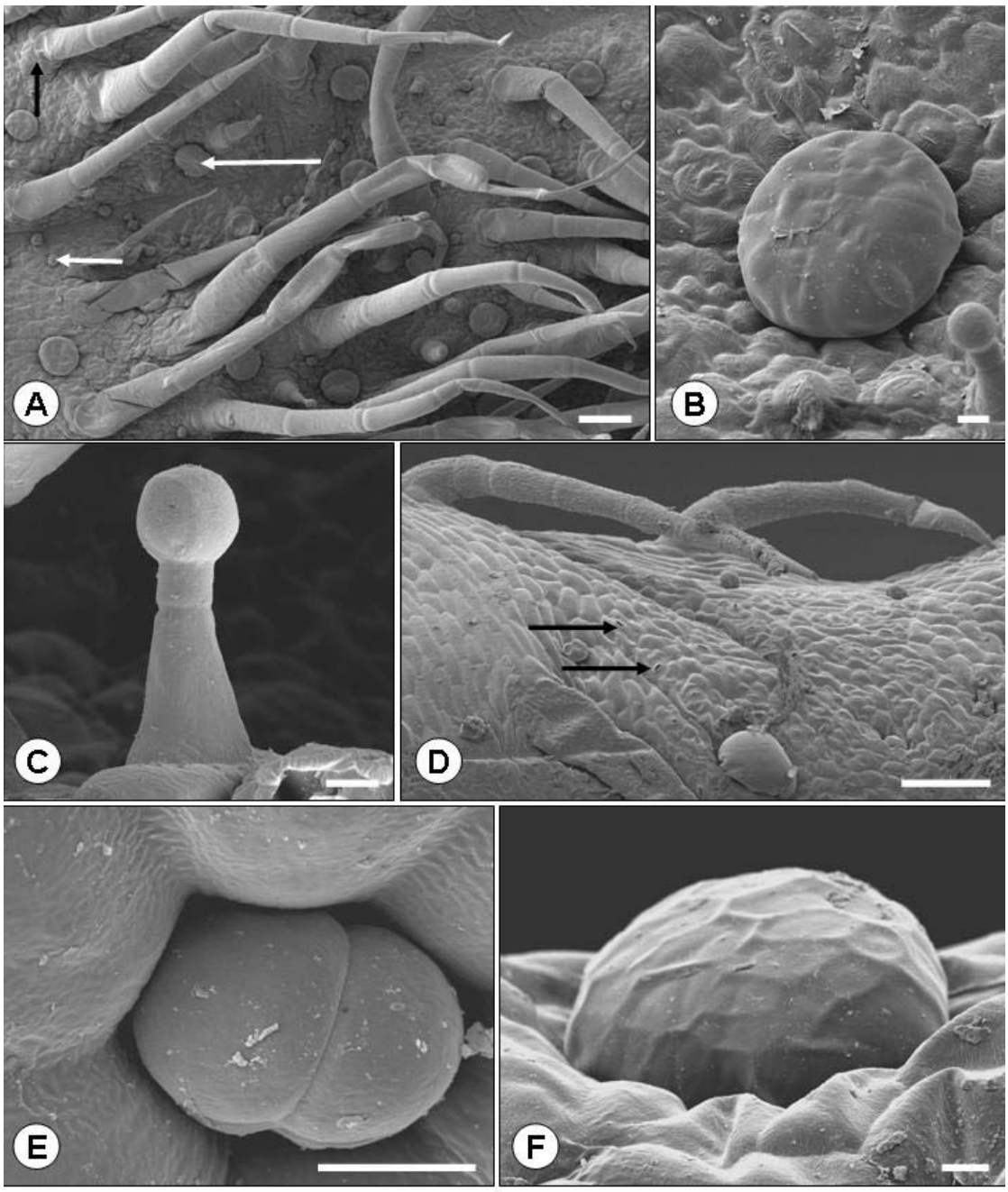


Figure 3.6 SEM micrographs of *Salvia lanceolata* (**A, B** and **C**) and *S. muirii* (**D, E** and **F**). **(A):** upper leaf surface showing capitate (short arrow) and peltate (long arrow) trichomes and relatively dense point shaped multicellular non-glandular trichomes, bar = 100 μm; **(B):** sessile peltate trichome covered by turgid and intact cuticle, bar = 10 μm; **(C):** peltate trichome and non-glandular trichomes with micropapillae (black arrow), bar = 10 μm; **(D):** upper surface with glandular and non-glandular trichomes, bar = 100 μm; **(E):** multicellular capitate trichome and multicellular non-glandular trichomes with micropapillae (black arrow), bar = 10 μm; **(F):** multicellular peltate trichomes with an intact cuticle, bar = 100 μm.



F**igure 3.7** SEM micrographs showing the types of trichomes found on the adaxial leaf surface of *Salvia repens* (**A, B** and **C**) and *S. schlechteri* (**D, E** and **F**). **(A):** capitate (short white arrow) and peltate (long white arrow) trichomes, the black arrow indicates a multicellular non-glandular trichome with many cells at the base, bar = 100 μm; **(B):** capitate trichome with stalk cells and peltate trichome covered by an intact cuticle, bar = 10 μm; **(C):** a capitate trichome with two stalk cells, bar = 10 μm; **(D):** glandular and very sparse multicellular non-glandular trichomes mainly at the vein, stomata visible (black arrows), bar = 100 μm; **(E):** capitate trichome with two secretory cells imbedded in a cavity, bar = 10 μm; **(F):** peltate trichome with intact cuticle imbedded in a cavity, bar = 10 μm.

3.3.2 Light microscopy observations

The presence of glandular and non-glandular trichomes was confirmed using light microscopy (LM). The general leaf structure in transverse section of each taxon is shown in Figures 3.8 to 3.13, while the various types of trichomes found in all species are illustrated in Figure 3.14. Micro-ornamentation was not evident. No variation in trichome types was found on leaves at different stages of development (mature and immature leaves). The trichome types of the species investigated are summarised in Table 3.1.

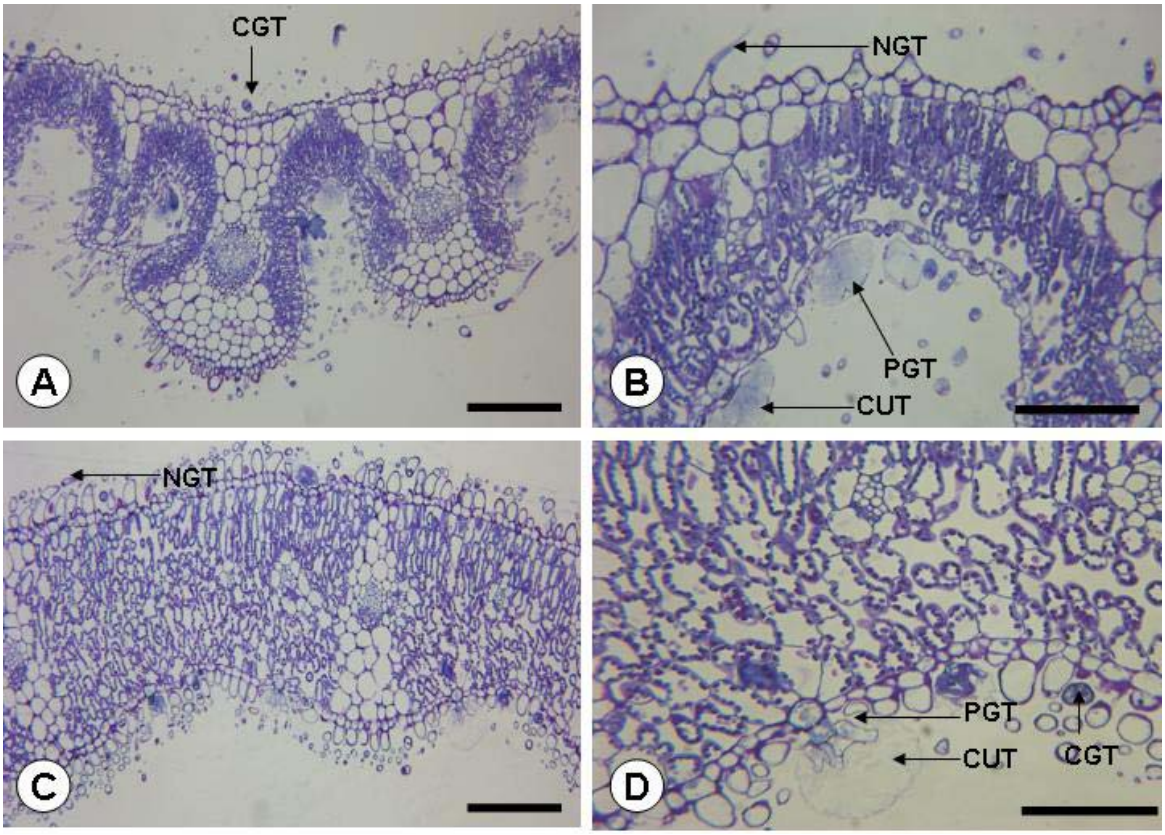


Figure 3.8 LM micrographs showing transverse sections of *Salvia africana-caerulea* (**A** and **B**) and *S. africana-lutea* (**C** and **D**). **(A):** capitate trichomes of mature leaf, bar = 0.2 mm; **(B):** mature leaf revealing the presence of glandular and non-glandular trichomes, bar = 0.1 mm; **(C):** young leaf showing non-glandular trichomes, bar = 0.2 mm; **(D):** young leaf showing glandular trichomes, bar = 0.1 mm. CGT: capitate trichome with head composed of one or more than one cell; CUT: cuticle of a peltate trichome; NGT: non-glandular trichome; PGT: peltate trichome (multicellular head).

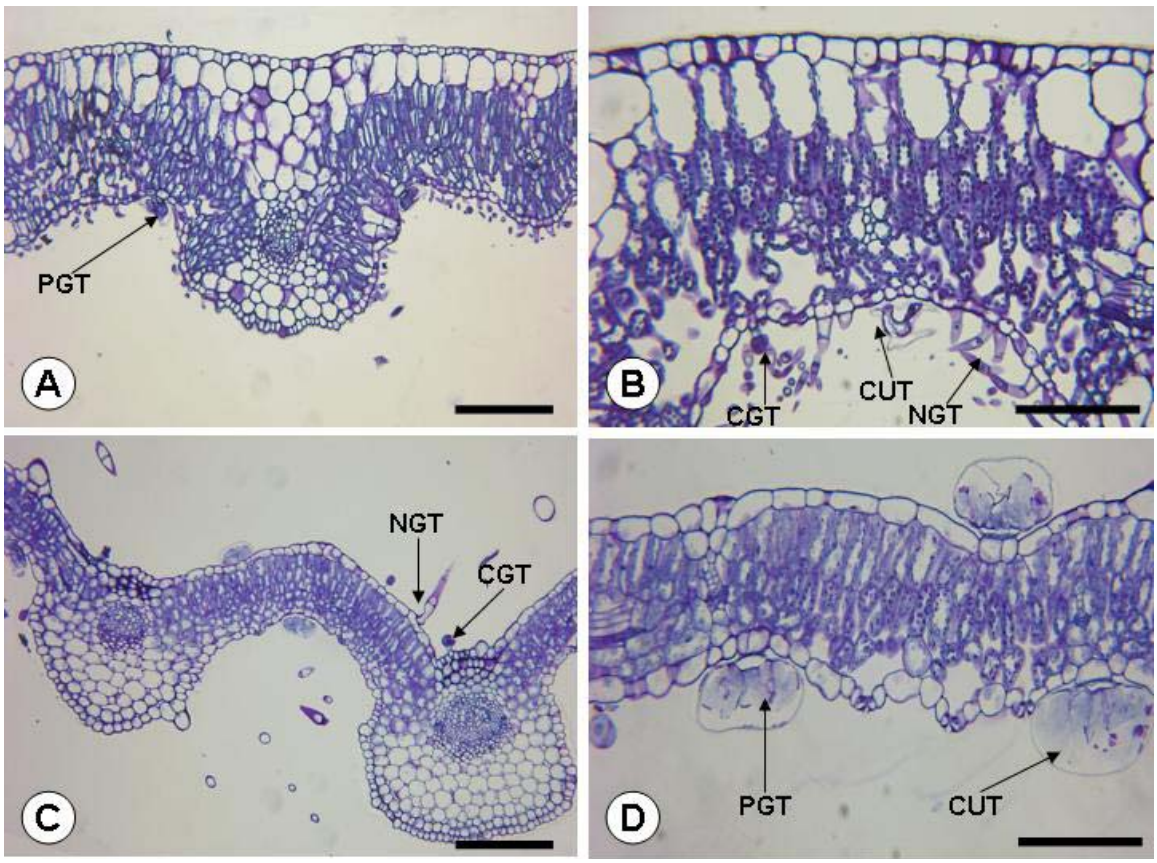


Figure 3.9 LM micrographs showing transverse sections of *Salvia albicaulis* (**A** and **B)** and *S. aurita* **C** and **D**). **(A):** mature leaf with peltate trichomes, bar = 0.2 mm; **(B):** mature leaf with glandular and non-glandular trichomes, bar = 0.1 mm; **(C):** mature leaf showing capitate and non-glandular trichomes, bar = 0.2 mm; **(D):** mature leaf revealing peltate trichomes and the cuticle, bar = 0.1 mm. CGT: capitate trichome with head composed of one or more than one cell; CUT: cuticle of a peltate trichome; NGT: non-glandular trichome; PGT: peltate trichome (multicellular head).

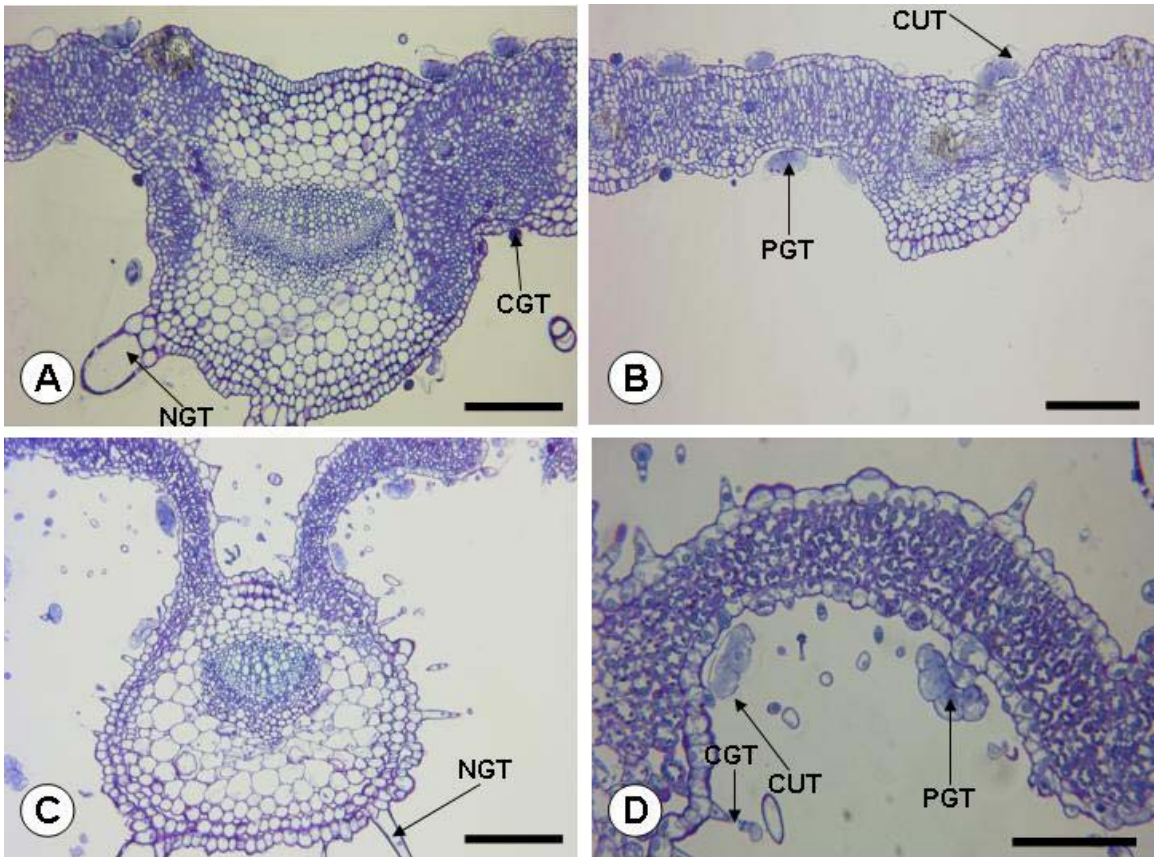


Figure 3.10 LM micrographs showing transverse sections of *Salvia chamelaeagnea* (**A** and **B)** and *S. disermas* (**C** and **D**). **(A):** mature leaf showing the general morphology, bar = 0.2 mm; **(B)**: young leaf with peltate trichomes, bar = 0.1 mm; **(C):** mature leaf revealing non-glandular trichomes, bar = 0.2 mm and **(D):** young leaf with glandular trichomes, bar = 0.1 mm. CGT: capitate trichome with head composed of one or more than one cell; CUT: cuticle of a peltate trichome; NGT: non-glandular trichome; PGT: peltate trichome (multicellular head).

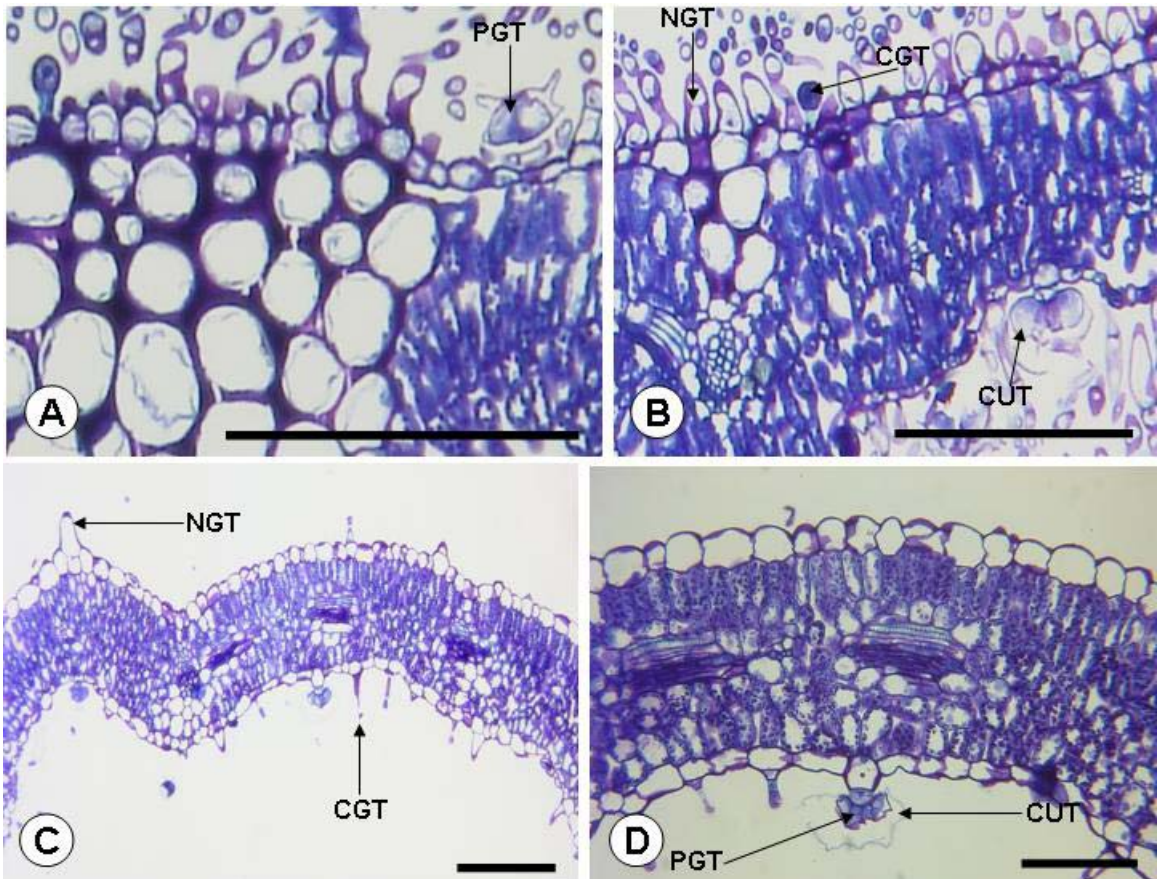


Figure 3.11 LM micrographs of transverse sections of *Salvia dolomitica* (**A** and **B)** and *S. garipensis* (**C** and **D**). **(A):** mature leaf showing a peltate trichome, bar 0.2 mm; **(B)**: young leaf showing non-glandular and glandular trichomes, bar = 0.1 mm; **(C):** mature leaf revealing the capitate and non-glandular trichomes, bar = 0.2 mm; **(D):** mature leaf revealing a peltate trichome, bar = 0.1 mm. CGT: capitate trichome with head composed of one or more than one cell; CUT: cuticle of a peltate trichome; NGT: non-glandular trichome; PGT: peltate trichome (multicellular head).

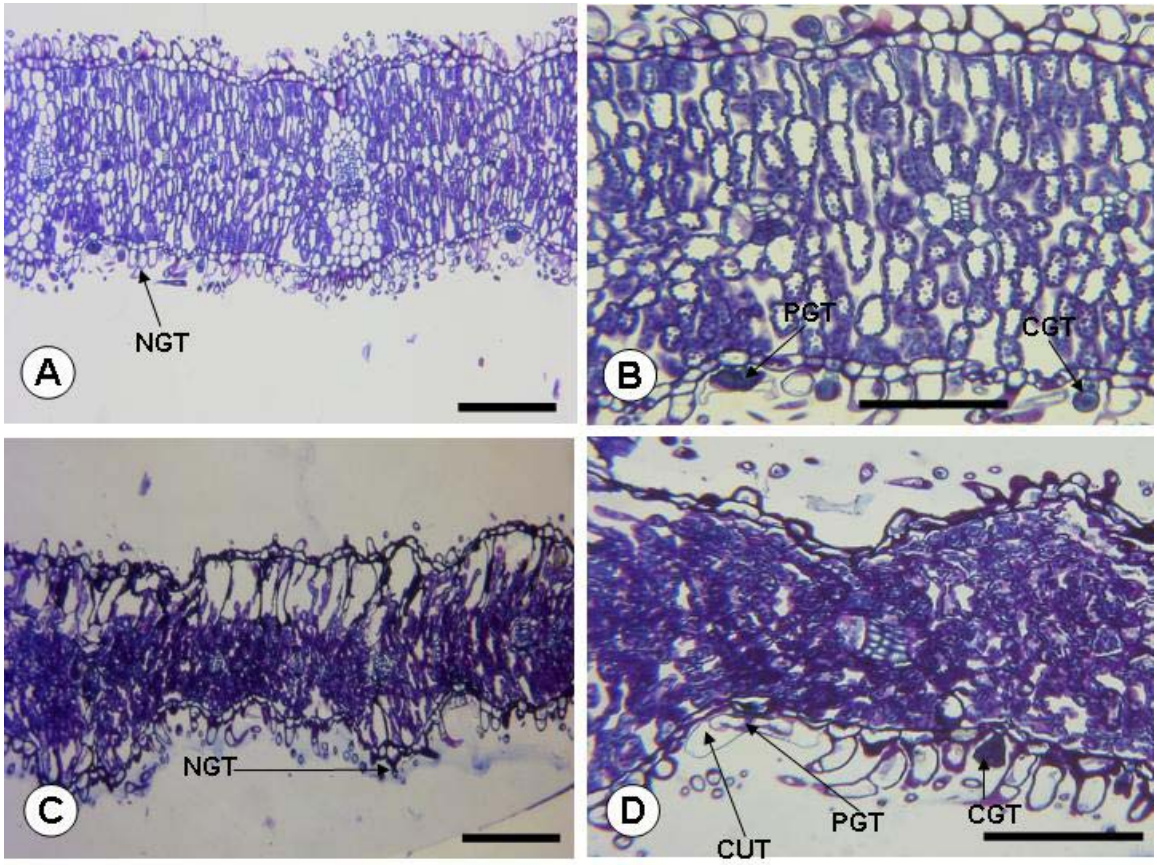


Figure 3.12 LM micrographs of transverse sections of *Salvia lanceolata* (**A** and **B)** and *S. muirii* (**C** and **D**). **(A):** mature leaf showing an abundance of non-glandular trichomes, bar = 0.2 mm; **(B)**: young leaf showing glandular trichomes, bar = 0.1 mm; **(C):** mature leaf revealing non-glandular trichomes, bar = 0.2 mm; **(D):** mature leaf showing various glandular trichomes, bar = 0.1 mm. CGT: capitate trichome with head composed of one or more than one cell; CUT: cuticle of a peltate trichome; NGT: non-glandular trichome; PGT: peltate trichome (multicellular head).

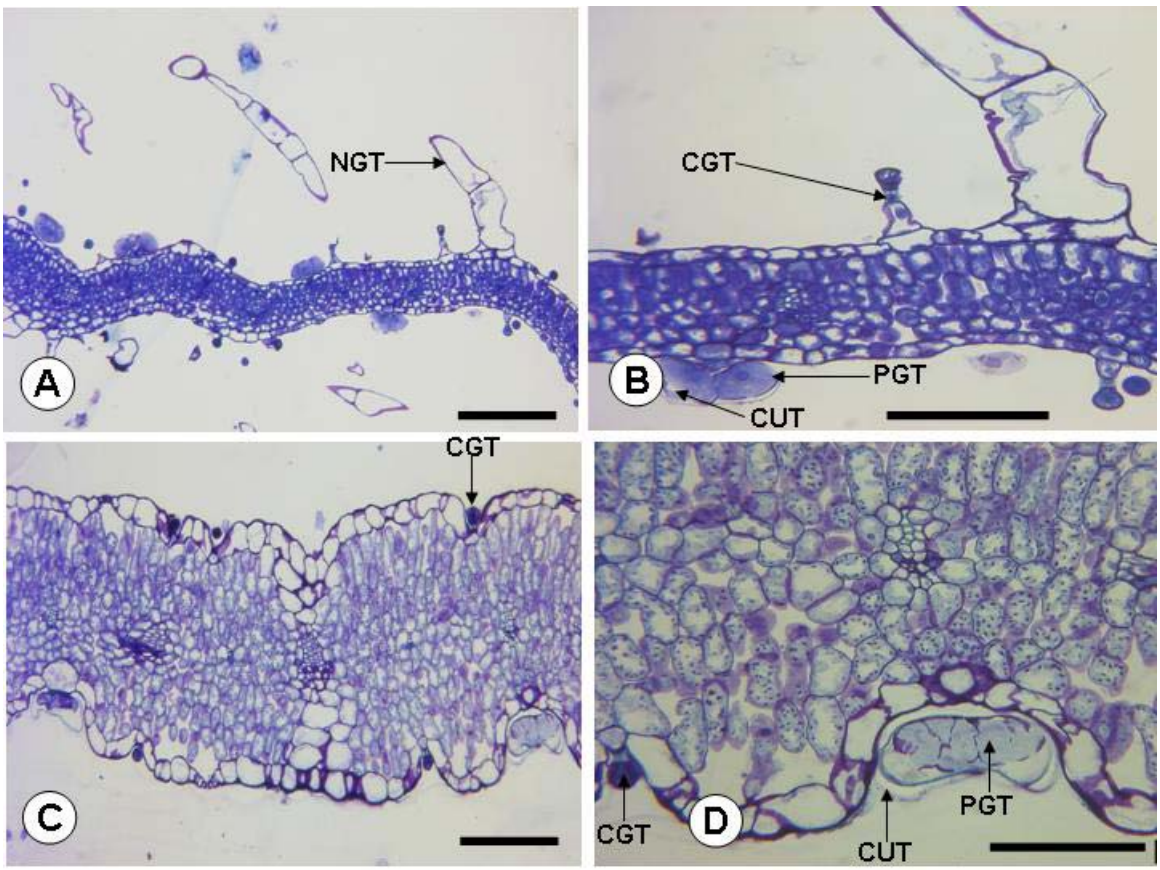


Figure 3.13 LM micrographs of transverse sections of *Salvia repens* (**A** and **B)** and *S. schlechteri* (**C** and **D**). **(A):** mature leaf showing non-glandular trichomes, bar 0.2 mm; **(B)**: capitate and peltate trichomes on a mature leaf, bar = 0.1 mm; **(C):** mature leaf revealing capitate trichomes, bar = 0.2 mm; **(D):** mature leaf showing glandular trichomes, bar = 0.1 mm. CGT: capitate trichome with head composed of one or more than one cell; CUT: cuticle of a peltate trichome; NGT: non-glandular trichome; PGT: peltate trichome (multicellular head).

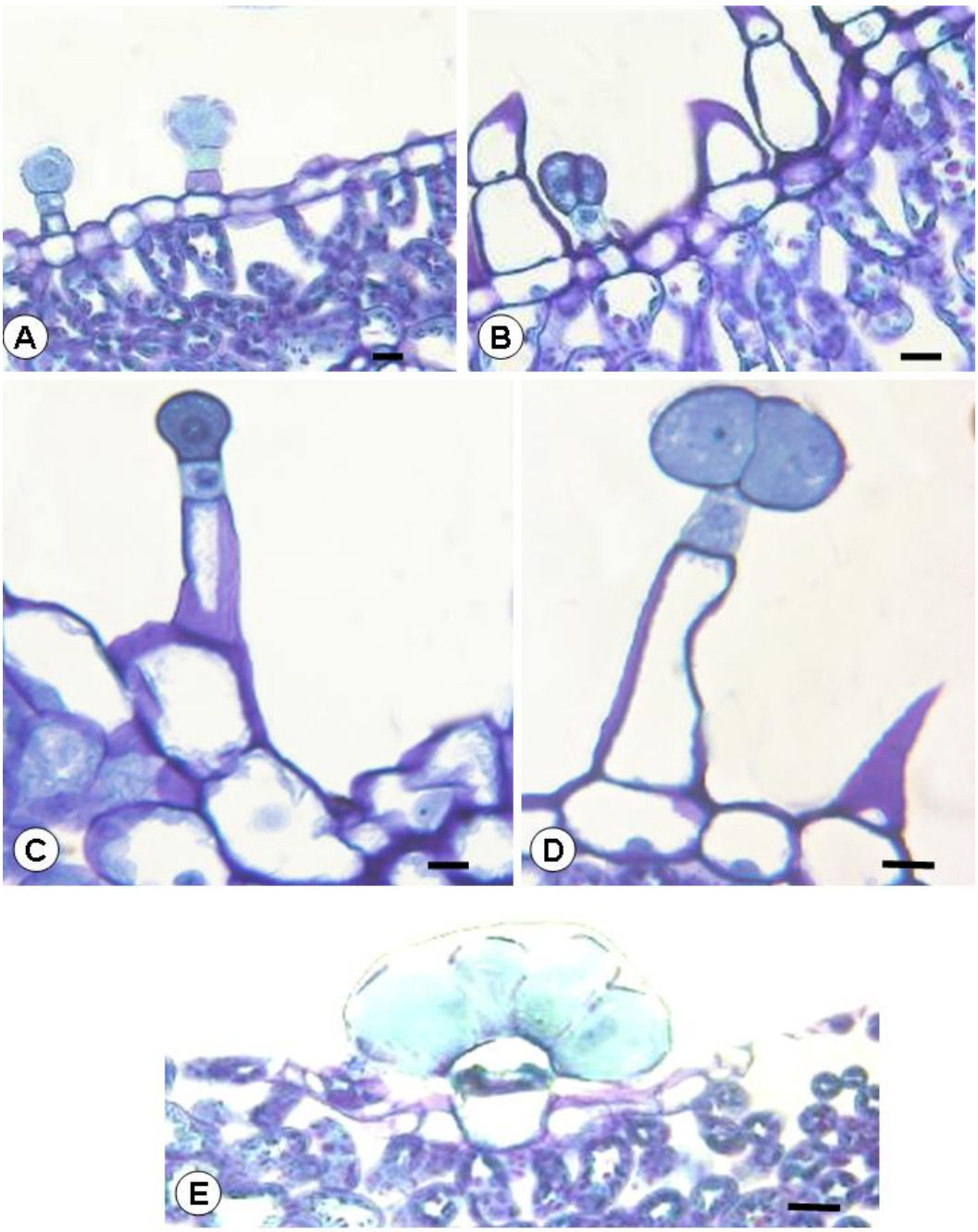


Figure 3.14 Various types of glandular and non-glandular trichomes found in *Salvia* species. **(A):** type I capitate trichomes of *S. africana-caerulea,* with unicellular head and short stalks, bar = 10 µm; **(B):** capitate trichome type II of *S. africana-lutea,* with head composed of more than one cell and short stalk, bar = 10 µm; **(C)**: capitate type III trichome of *S. garipensis,* with unicellular head and long stalks, bar = 10 µm; **(D):** capitate trichome type IV of *S. garipensis,* with head composed of more than one cell and long stalk, bar = 10 µm; **(E):** peltate trichome of *S. africana-caerulea* with up to sixteen secretory head cells and short stalk, bar = 10 µm.

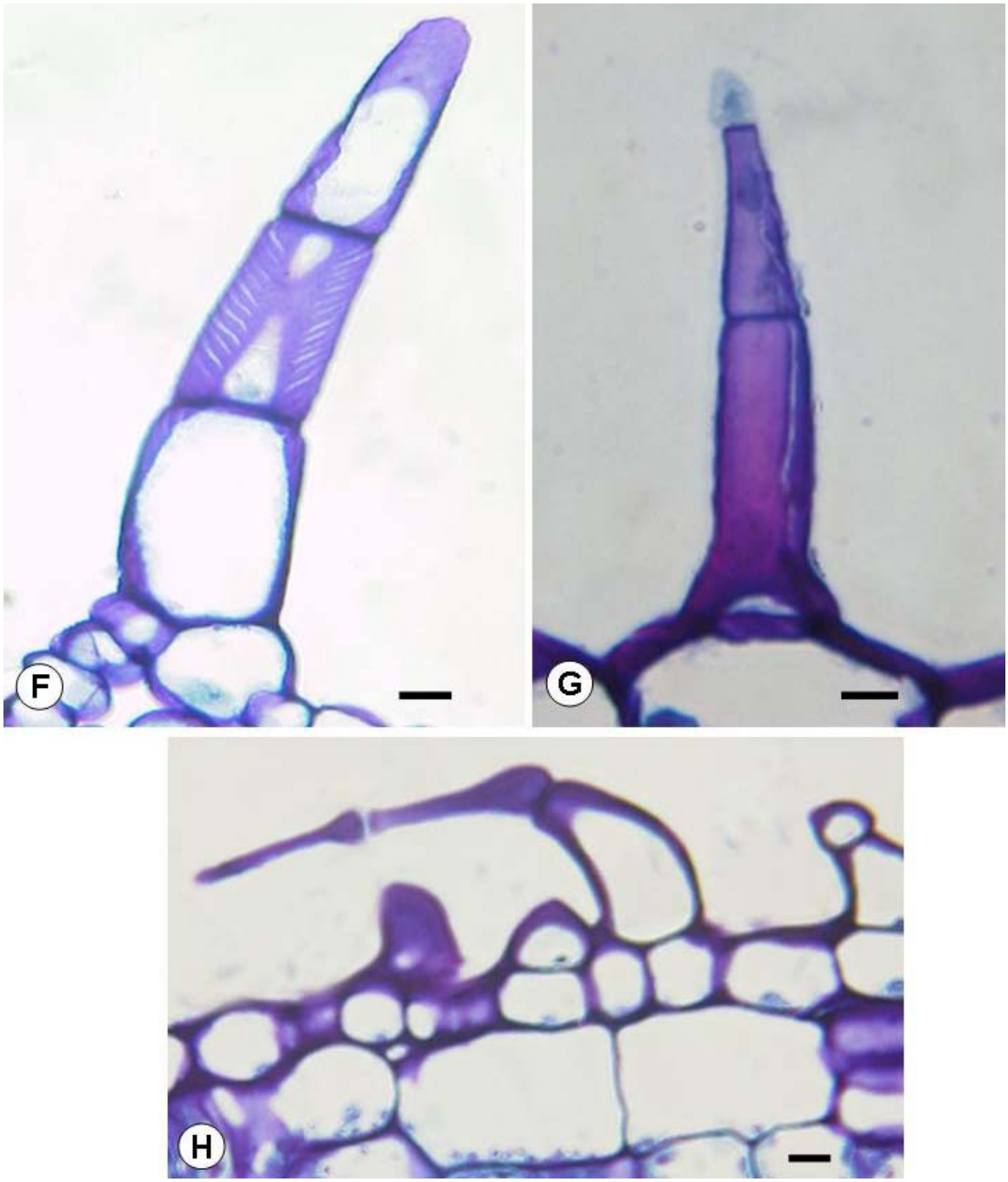


Figure 3.14 Contd. Various types of glandular and non-glandular trichomes found in *Salvia* species: **(F):** non-glandular trichome with round top (*S. aurita*), bar = 10 µm; **(G):** short non-glandular trichome composed of two cells (*S. dolomitica*), bar = 10 µm; **(H):** sharply-pointed non-glandular trichome (*S. africana-caerulea*) composed of three cells, bar = 10 µm.

Table 3.1 Distribution of trichomes on the leaves of *Salvia* species: absence (–) and presence (+) based on trichome type observations in 3.14.

Species	Accession number (KBG	A	B	C	D	E	F	G	H
S. africana-caerulea	0554/82	+	–	–	–	+	–	–	+
S. africana-lutea	1185/71	+	+	–	–	+	–	–	+
S. albicaulis	0050/79	+	+	–	–	+	–	–	+
S. aurita	0244/01	+	+	–	–	+	+	–	–
S. chamelaeagnea	0725/93	–	+	–	–	+	–	–	+
S. disermas	0574/74	+	+	+	–	+	–	–	+
S. dolomitica	0038/79	+	–	–	–	+	–	+	–
S. garipensis	0689/91	+	+	+	+	+	–	–	+
S. lanceolata	0919/96	+	+	–	–	+	–	–	+
S. muirii	189/82	–	+	–	–	+	–	+	–
S. repens	0609/95	+	–	+	–	+	+	–	–
S. schlechteri	0173/02	–	+	–	–	+	+	–	–

(A): Capitate trichomes: single-celled head, two or three short stalk cells, (B): capitate trichome: two-celled head, two or three short stalk cells, (C): capitate trichomes: single-celled head, two or three long stalk cells, (D): capitate trichomes: two celled-head, two or three long stalk cells, (E): peltate trichomes: four to sixteen cells in the head, one or two stalk cells, (F): non-glandular trichome with round top, (G): short non-glandular trichome composed of two cells, (H): sharply-pointed non-glandular trichome composed of three cells.

The capitate trichome type varies in the number of secretory cells (up to four cells) with one to four stalk cell(s) and one basal epidermal cell. To accommodate differences in the number of cells comprising the head and the length of the stalk cells, capitate trichomes were classified into four types. Capitate trichome type I refers to a structure composed of a unicellular head, a short stalk cell and one basal epidermal cell. The head of the capitate trichome type II is composed of more than one cell with short stalk cell(s) and one basal epidermal cell. Type III capitate trichome is composed of a unicellular head, a long stalk cell(s) and one basal epidermal cell and type IV capitate trichome comprises a head with more than one cell, a long stalk cell(s) and one basal epidermal cell (Figure 3.14A-D). In general, the head of a capitate trichome has a slightly greater diameter than that of the stalk cells. The types of capitate trichomes found in different species are recorded in Table 3.2.

Table 3.2 Various types of capitate trichomes observed in *Salvia* species.

Trichome type	Characteristics	*Salvia* species
I	Unicellular head, short stalk, one basal epidermal cell	*S. africana-caerulea, S. africana-lutea, S. albicaulis, S. aurita, S. dolomitica, S. disermas, S. garipensis, S. lanceolata, S. repens*
II	Head with more than one cell, short stalk, one basal epidermal cell	*S. africana-lutea, S. albicaulis, S. aurita, S. chamelaeagnea, S. disermas, S. lanceolata, S. garipensis, S. muirii,S. schelchteri*
III	Unicellular head, long stalk, one basal epidermal cell	*S. disermas, S. garipensis, S. repens*
IV	Head with more than one cell, long stalk, one basal epidermal cell	*S. garipensis*

Peltate glandular trichomes consist of a round broad multicellular head with up to sixteen cells, a short stalk cell(s) with cutinized outer walls and a basal epidermal cell (Figure 3.14E). In the species examined here, the secretory cells of the head are arranged in one (Figure 3.3B) or two (Figure 3.3F) concentric circles. The number of cells in the central circle is usually four, while in the periphery the cell number varies according to the species [e.g. 10 peripheric cell in *S. aurita* (Figures 3.3F) and 12 peripheric cells in *S. chamelaeagnea* (Figure 3.4B)]. The stalk cell(s) of the peltate trichomes are often sunken to various degrees into grooves in the epidermis (Figures 3.4A, 3.6F and 3.7F).

The non-glandular trichomes are uniseriate, unbranched and multicellular and may vary in shape and number of cells (up to seven) among species. However, a stalk composed of 4 or 5 cells is the most common type and was found in the majority of species (Table 3.1). For most species, the base of the non-glandular trichomes is composed of one cell, however, multiple cells at the base of the non-glandular trichomes could be seen in *S. albicaulis*, *S. aurita* and *S. repens*. The various groups of non-glandular trichomes are illustrated in Figure 3.14F-H.

The distribution (presence/absence) of the various trichome types found in each investigated species is listed in Table 3.1. Based on SEM and LM observations, the species studied can be arranged into six groups (Table 3.2).

The relative abundance of glandular trichomes follows the increasing order: *S. disermas* > *S. garipensis* > *S. africana-caerulea* > *S. chamelaeagnea* > *S. aurita* > *S. schlechteri* > *S. repens* > *S. africana-lutea*, while *S. dolomitica*, *S. albicaulis*, *S. muirii* and *S. lanceolata* have a lower concentration of glandular trichomes.

Although the trichome types found vary between species, the use of trichomes alone could not be used to delimit one species from another. Other characters should also be considered.

3.4 Discussion

In the Lamiaceae (Labiatae), many species are densely covered with both glandular and non-glandular hairs (Metcalfe and Chalk, 1950; Serrato-Valenti *et al*., 1997; Bisio *et al*., 1999). Two types of glandular trichomes generally occur together in a single leaf, viz. capitate and peltate, the latter being the most characteristic of the family (Metcalfe and Chalk, 1950). The capitate trichomes of *Salvia* species investigated varied and were designated type I, II, III or IV (Figure 3.14A-D). An SEM study of the *Salvia stenophylla* complex indigenous to southern Africa, similarly revealed the presence of non-glandular and glandular trichomes with the latter being capitate or peltate (Gono-Bwalya, 2003). Investigation of the various organs of *S. glutinosa* L. revealed that glandular and non-glandular trichomes were present (Kaya *et al*., 2003). In this study, the SEM and LM observations revealed the presence of both these trichome types on the adaxial and abaxial surfaces of each species (Figures 3.2 to 3.7 and 3.14A-E). Similar glandular trichome types were found in most species studied. However, taking into account the variation that exists in the nature of the stalk (long or short) and in the number of secretory cells in the head, the species investigated could be divided into four distinct groups (Table 3.2).

The taxonomic significance of the morphology of glandular hairs in members of the Lamiaceae is not yet determined and it is still being debated in the literature (Falciani *et al*., 1995; Fahn, 2001). Generally in this family, capitate trichomes are unicellular or

multicelullar with short or long stalk cells and one basal epidermal cell. The peltate trichomes are composed of one to sixteen cells and a short stalk cell(s) and one basal epidermal cell (Metcalfe and Chalk, 1950; Hallahan, 2000). The cells of the heads of peltate trichomes are usually arranged in two concentric circles and the number of cells in the central circle is usually four. Werker *et al*. (1985) investigated the glandular hairs of eight species of the Lamiaceae and found that the central circle was composed of four cells, while the number of cells on the periphery varied from 6 to 14 cells. In the current study, the peltate trichomes are composed of one or two circles, the central circle being composed of four cells, while the number of cells in the periphery varied from 6 to 12 cells (Figures 3.3F and 3.4B) which concurs with previous studies of the Lamiaceae.

There is a discrepancy in the literature regarding the type of glandular trichomes which are widespread in the Lamiaceae, in particular, the genus *Salvia*. Some authors regard the peltate trichome type as the most common in *Salvia* species (Metcalfe and Chalk, 1950; Werker *et al*., 1985) while others consider, a peltate trichome with a single-celled stalk and the capitate trichome with a unicellular head to be the most common (Hanlidou *et al*., 1991). Metcalfe and Chalk (1950) also state that heads with one or two cells are present in capitate trichomes of *Salvia*. However, Werker *et al*. (1985) pointed out that the bicellular head was rather rare. Bourett *et al*. (1994) also observed small capitate glandular trichomes with two secretory cells, which were similar to those found in the current study (Figure 3.14B). In this study, unicellular, bicellular or even four-celled heads (with short or long stalk cells) were present in all 12 species (Table 3.1). On the basis of external morphology, the glandular trichomes of the species studied are similar to those previously described in the Lamiaceae, namely *S. glutinosa* L. and *S. africana-lutea* (Serrato-Valenti *et al*., 1997; Kaya *et al*., 2003).

Non-glandular trichomes in Lamiaceae may be unicellular or multicelullar with up to seven cells in *Salvia* species (Werker *et al*., 1985). This is consistent with the observations recorded here, with up to six cells being observed in some species (Figure 3.7A). Trichome types reported in this study have been identified in exotic species such as *S. glutinosa* and *S. officinalis*.

It is important to note that trichome morphology can vary between the plant organs (leaf, stem or calyx) studied in addition to the stage of development. However, in this

investigation, no variation could be observed between the mature and the immature plant material regarding the trichome types. It was shown in *S. glutinosa* that the presence or absence of various trichome types, as well as their density varied for different plant organs (Kaya *et al*., 2003). Changwe (2002) also observed that glandular trichomes, like non-glandular ones on the leaves, stems and calyces, are unevenly distributed in *Thorncroftia* species. An investigation of other organs in the investigated species may well reveal similar variation, but was beyond the scope of this study.

Studies have shown that the short-stalked glands with up to sixteen or more secretory cells in the head secrete pleasantly scented oils such as those of *Salvia* and other aromatic plants e.g. *Rosmarinus officinalis* and *Thymus vulgaris* (Metcalfe and Chalk, 1950). *Prasium majus* (Lamiaceae) which is not aromatic only possesses capitate trichomes (Werker *et al*., 1985). It is possible to isolate an individual gland from the leaf surface and analyse its contents for chemical composition and presence of specific oils or enzymes (Svoboda and Svoboda, 2000). This study was however not extensive enough to determine whether the glandular hairs produce the fragrance of sage would be interesting to determine. Glandular trichomes contain essential oils as well as other secretions that can cover the surrounding surface of the leaves. Some trichomes have specifically evolved to act as defense mechanisms. Trichomes that occur in large numbers may protect a plant by making its tissue less edible or inhospitable to feeding and breeding insects (Harbone, 1993).

Observations made in this study suggest that the secreted material in *Salvia* species can be released by breaking the cuticle or via pores in the cuticular structures (Figure 3.4B and 3.14A) (Serrato-Valenti *et al*., 1997; Bisio *et al*., 1999). While the first mode is common to many aromatic species, the second has only been described for capitate trichomes in the members of the Lamiaceae (Figure 3.14A) (Werker *et al*., 1985).

The outer surface of the non-glandular trichomes sometimes exhibits micro-ornamentation, which may show great diversity causing it to appear micropapillate (Werker, 2000). Micro-ornamentations were not evident in this study. However, micropapillae were only visible under high magnification in the SEM study on the non-glandular trichomes of some species including *S. chamelaeagnea*, *S. lanceolata* and *S. muirii* (Figure 3.4A, 3.6A and 3.6E, respectively). An expanded cuticle of the peltate trichomes at maturity has been reported in other studies in the Lamiaceae (Hallahan, 2000). This cuticle could be seen in

all species investigated (e.g. Figure 3.3 B, 3.3F and 3.4B). It is probably through the rupture of the cuticle that secondary metabolites such as essential oils contained in the heads are released.

Non-glandular trichomes are also frequent in the Lamiaceae, and may be uniseriate, tufted or branched (Metcalfe and Chalk, 1950; Werker *et al.,* 1985; Kaya *et al*., 2003). In this study, they were found to be uniseriate and unbranched (Figure 3.14F-H) and the major difference was the number of cells at the base rather than the number of cells forming the stalk. The basal cells of the non-glandular hairs can be used to distinguish *S. aurita*, *S. chamelaeagnea* and *S. repens* from other species. While glandular hairs act as a chemical defense against herbivores or insects, non-glandular hairs constitute a physical defense of the plant (Werker *et al*., 1985). The distribution of non-glandular trichomes is variable. For some species (*S. africana-lutea*, *S. dolomitica* and *S. muirii*), they might form a dense cover; while in *S. aurita*, *S. chamelaeagnea* and *S. schlechteri*, they are sparse (Figure 3.2C, 3.3D, 3.4A, 3.5A, 3.6D and 3.7D).

The reliability of trichomes as taxonomic characters varies from one group of plants to another and may be influenced by many factors such as the plant parts studied and the stage of development of the plant. Trichome characteristics are regarded as being under strong genetic control and less little affected by the environment (Mueller, 1966; Cutler and Brandham, 1977). But, Stace (1965) and Dilcher (1974) suggest that the characteristics might vary according to environmental factors such as soil type and climate. Furthermore, some attributes of the leaf may have arisen in response to evolutionary pressures. The question has often been posed whether the density of the glandular hairs is fixed during the life span of the leaf or increases with age. Some authors have suggested that the number is established early during the leaf differentiation (Ascensâo and Pais, 1987), while others have concluded that it increases throughout all stages of leaf development (Maffei *et al*., 1989). Taking into consideration these observations, the density of trichomes should be recorded carefully. This is also true with the number of cells in capitate or peltate trichomes, since the number of cells present at a particular stage may increase during a later stage of development (Ascensâo and Pais, 1987).

Substances such as polysaccharides, essential oils, salts, sugars and proteins are secreted by glandular trichomes in species of the Lamiaceae (Werker *et al*., 1985; Karousou *et al*.,

1992; Ascensâo *et al*., 1995). The study of trichomes demonstrated that the species investigated could be divided into groups, but distinction between species was not possible. The composition of the essential oils (Chapter 4) in addition to leaf trichome types observed in *Salvia* species may facilitate the identification of *Salvia* species.

3.5 Conclusions

- Two types of glandular trichomes (peltate and capitate) were observed to be present in all 12 species.
- The peltate trichome type is present in all species but varies in the number of head cells (four to sixteen).
- Similarly, the capitate trichomes vary in the number of head cells and the length of the stalk cells.
- As with other members of the Lamiaceae, species of the genus *Salvia* showed some degree of diversity in leaf trichome types, but not sufficient to distinguish between the investigated species.
- *Salvia aurita*, *S. chamelaeagnea* and *S. repens* have multiple cells at the base of the non-glandular trichomes, unlike the rest of species only one basal cell.
- Based on the type of capitate trichomes, the species investigated could be divided into four distinct groups.

Chapter 4: Essential Oil Composition

Abstract

Eleven of the seventeen species under investigation are pungently aromatic which prompted a study of their essential oils. The essential oils were isolated by hydrodistillation and analyzed by GC and GC-MS. The oil yield was relatively low and ranged between 0.004 (*S. radula*) to 0.50% (*S. muirii*) (w/w). Ninety-three components were identified representing 72.9 to 97.5% of the oils. Quantitative and qualitative variations in oil composition were observed among species. The essential oils were dominated by monoterpene hydrocarbons in *S. muirii* (53.0%), *S. stenophylla* (41.5%), *S. africana-lutea* (35.6%) and *S. repens* (32.6%). Oxygen-containing monoterpenes dominated in *S. dolomitica* (71.8%) and *S. chamelaeagnea* (42.8%), while oxygen-containing sesquiterpenes were dominant in the remaining samples. Major components identified include α-pinene, 1,8-cineole, linalool, limonene, myrcene, β-caryophyllene, spathulenol, β-caryophyllene oxide, viridiflorol, δ-3-carene and α-bisabolol. Quantitative and qualitative heterogeneity in the oils was supported by the essential oil cluster analysis. Only *S. africana-caerulea* and *S. lanceolata* showed a very high correlation ($S_{corr} > 0.9$).

4.1 Introduction

Essential oils are odorous, volatile products of an aromatic plant's secondary metabolism normally formed in special cells, or groups of cells, such as trichomes (Metcalfe and Chalk, 1950; Werker *et al*., 1985). They are generally concentrated in one particular region of a plant such as leaves, stems, fruits, barks or roots and when they occur in different organs in the same plant, they frequently have different composition profiles (Araújo *et al*., 2003).

Despite the strong competition from synthetic products, essential oils currently still occupy an important place in the perfume, fragrance and therapeutic (mainly aromatherapy) industries (Lis-Balchin *et al*., 1998). Many aromatic plants are also used in traditional medicine for various purposes (Cimanga *et al*., 2002). The total value of the essential oil industry worldwide was estimated at about 10 billion US dollars with production from the developing countries representing about 55% of world production (IDRC, 2006). Aromatherapy is the therapeutic use of fragrances, or at least volatiles, to cure, mitigate or prevent disease, infection and indisposition by means of inhalation (Buchbauer, 2000). Essential oils from aromatic and medicinal plants have been known to possess biological activity since time immemorial. There are many reports describing the pharmacological properties of essential oils (Baratta *et al*., 1998; De Feo *et al*., 2003). This has recently attracted the attention of many scientists and encouraged them to screen plants for the pharmacological properties of their oils. With the growing interest in the use of essential oils in perfume and pharmaceutical industries, a systematic examination of plants for these properties has become increasingly important.

Studies on the chemical composition of essential oils have proven to be a valuable tool in the resolution of some taxonomic problems and can help in determining the relationship between different species in the genus. The study of essential oils is important not only from a taxonomic view point, but also from the pharmacological aspect, as plants with similar chemical compositions may theoretically have very few differences in their biological properties.

The objectives of this study were to:

(i) determine the chemical composition of 11 essential oils using GC and GC-MS analysis, and

(ii) determine any possible chemotaxonomic relationship between the investigated species.

4.2 Materials and methods

The essential oils were isolated by hydrodistillation as explained in Section 2.3.1 (Figure 2.1) and were analyzed using gas chromatography (Figure 4.1a) and gas chromatography coupled to mass spectrometry (Figure 4.1B) (Pedro *et al.,* 2001).

4.2.1 Gas chromatography

GC analyses were performed using a Perkin Elmer 8700 gas chromatograph equipped with two FIDs, a data handling system and a vaporizing injector port into which two columns of different polarities were installed: a DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific Inc., Rancho Cordova, CA, USA) and a DB-17 HT fused-silica column (30 m x 0.25 mm i.d., film thickness 0.15 μm; J & W Scientific Inc.).

Figure 4.1 Gas chromatography (**A**) and gas chromatography coupled to mass spectrometry (**B**) apparatus.

The oven temperature was programmed to run from 45-175 °C, at 3 °C/min, then to increase in temperature by 15 °C/min up to 300 °C, where it was maintained for 10 min; injector and detector temperatures, 280 °C and 290 °C, respectively; carrier gas, hydrogen, adjusted to a linear velocity of 30 cm/s. The samples were injected using the

split sampling technique, ratio 1:50. The percentage composition of each oil sample was computed from the GC peak areas using the normalization method; the data were calculated as mean values of two injections from each oil sample without using response factors.

4.2.2 Gas chromatography coupled to mass spectrometry

The gas chromatography coupled to mass spectrometry (GC-MS) unit (Figure 4.1B) consisted of a Perkin Elmer Autosystem XL gas chromatograph, equipped with DB-1 fused-silica column (30 m x 0.25 mm i.d., film thickness 0.25 μm; J & W Scientific, Inc.), and interfaced with Perkin-Elmer Turbomass mass spectrometer (software version 4.1). Oven temperature was as above; transfer line temperature, 280 °C; ion trap temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; ionization current, 60 μA; scan range, 40-300 u; scan time, 1 s.

4.3 Data analysis and chemotaxonomic relationship among species

The identity of the components was assigned by comparison of their retention indices relative to C_8-C_{17} *n*-alkanes and GC-MS spectra with corresponding data of components of reference oils, laboratory-synthesized components and commercially available standards from a home-made library from the Departamento de Biologia Vegetal, Universidade de Lisboa (Portugal). The percentage composition of the essential oil samples was used to determine the relationship between the different samples of *Salvia* species by cluster analysis using the NTSYS software (Rohlf, 1992). Correlation was selected as a measure of similarity and the unweighted pair-group method with arithmetic average was used for cluster definition. The degree of correlation was evaluated according to Pestana and Gageiro (2000) where a very high correlation ranged between 0.90 and 1.00, high between 0.70 and 0.89, moderate between 0.40 and 0.69, low between 0.20 and 0.30 and very low if less than 0.20.

4.4 Results

4.4.1 Percentage yield

The essential oil yields among the 11 species ranged from 0.004 to 0.50% (w/w) wet biomass (Figure 4.2). The highest yield was obtained for *S. muirii*, while *S. radula* gave the lowest yield.

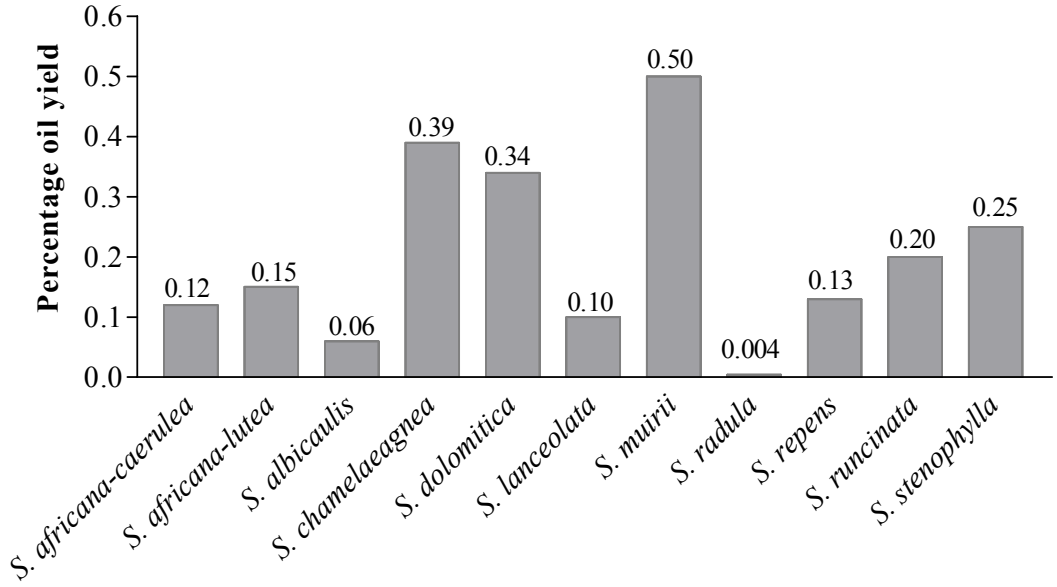


Figure 4.2 Percentage essential oil yields (w/w, wet biomass) obtained for eleven indigenous *Salvia* species.

4.4.2 Essential oil composition

The essential oil compositions determined by GC and GC-MS revealed 93 identified components present in the 11 species representing from 72.9% to 97.5% of the total oil composition. The components identified, together with their respective percentage composition in each species, are shown in Table 4.1, in order of their elution in a DB-1 column. The essential oils with high levels of monoterpene hydrocarbons include; *S. muirii* (53.0%), *S. stenophylla* (41.5%), *S. africana-lutea* (35.6%) and *S. repens* (32.6%) (Table 4.1). Oxygen-containing monoterpenes dominated in *S. dolomitica* (71.8%) and *S. chamelaeagnea* (41.8%), while the oils of the remaining species are dominated by oxygen-containing sesquiterpenes and include *S. africana-caerulea*, *S. albicaulis*, *S. lanceolata*, *S. radula* and *S. runcinata* (Table 4.1).

Table 4.1 Percentage of the components identified in the essential oils of 11 South African *Salvia* species.

Components	RI	Sac	Sal	Sab	Sch	Sdo	Sla	Smu	Sra	Sre	Sru	Sst
Tricyclene	921	-	tr	-	-	tr	-	0.1	-	-	-	0.1
α-Thujene	924	0.1	0.6	tr	0.2	-	0.5	0.3	-	4.7	-	0.1
Benzaldehyde	927	-	-	-	-	-	-	-	-	-	tr	tr
α-Pinene	**930**	**4.1**	**6.0**	**1.9**	**10.4**	**tr**	**2.6**	**22.3**	**-**	**tr**	**tr**	**1.3**
Camphene	938	0.1	tr	tr	0.2	-	tr	7.6	-	3.3	tr	2.0
Sabinene	958	tr	0.9	tr	0.1	0.1	1.4	0.1	-	2.4	tr	0.4
6-Methylhept-5-en-2-one	960	-	-	-	-	-	-	-	-	-	-	tr
1-Octen-3-ol	961	tr	0.9	tr	0.1	0.1	1.4	tr	0.2	tr	tr	-
β-Pinene	963	1.3	0.2	tr	1.9	tr	1.0	6.7	0.2	tr	0.1	0.2
2-Octanone	967	-	-	-	-	-	-	-	0.8	-	-	-
3-Octanol	974	-	-	-	-	-	-	-	-	-	0.1	-
Myrcene	**975**	**0.2**	**11.5**	**tr**	**1.6**	**0.6**	**0.6**	**1.9**	**-**	**1.0**	**0.1**	**3.9**
δ-2-Carene	983	-	-	tr	-	-	-	-	-	0.9	-	-
α-Phellandrene	995	-	-	-	-	-	-	-	-	-	-	0.1
δ-3-Carene	**1000**	**-**	**-**	**tr**	**-**	**-**	**-**	**-**	**-**	**1.4**	**-**	**22.6**
o-Ocimene	1000	-	-	-	-	-	-	-	-	-	-	0.3
p-Cymene	1003	1.5	7.6	2.5	0.9	0.2	1.1	1.6	0.2	9.5	tr	0.6
1,8-Cineole	**1005**	**0.5**	**1.9**	**9.4**	**40.5**	**tr**	**1.8**	**23.2**	**0.2**	**12.8**	**tr**	**-**
β-Phellandrene	1005	-	-	-	-	-	-	-	-	-	tr	3.7
Limonene	**1009**	**1.5**	**1.6**	**9.4**	**9.7**	**0.6**	**1.8**	**11.6**	**-**	**9.4**	**0.5**	**4.8**
cis-β-Ocimene	1017	tr	5.4	-	0.3	0.6	tr	0.5	-	-	0.1	-
trans-β-Ocimene	1027	tr	1.8	-	0.4	-	tr	0.1	-	-	-	0.2
γ-Terpinene	1035	-	-	-	-	tr	-	0.1	-	-	-	0.2
trans-Sabinene hydrate	1037	tr	tr	-	0.1	-	tr	-	-	-	-	0.5
cis-Linalool oxide	1045	-	-	-	-	0.2	-	-	-	-	-	-
n-Octanol	1045	-	-	-	-	-	-	-	-	-	0.1	-
trans-Linalool oxide	1059	-	-	-	-	0.2	-	-	-	-	-	-
Terpinolene	1064	-	-	-	-	0.2	-	0.1	-	-	-	1.0
cis-Sabinene hydrate	1066	0.1	tr	-	0.1	-	tr	-	-	-	-	-
Linalool	**1074**	**0.3**	**0.8**	**tr**	**0.6**	**16.6**	**1.3**	**-**	**-**	**tr**	**0.2**	**0.4**
trans-p-2-Menthen-1- ol	1074	0.6	0.2	tr	0.3	-	0.2	-	-	-	-	-
α-Campholenal	1088	-	-	tr	-	-	-	-	-	-	-	-
Camphor	1095	-	-	2.3	-	-	-	8.3	0.3	5.3	0.9	3.2
neo-iso-Thujanol	1101	-	-	tr	-	-	-	-	-	-	-	-

Components	RI	Sac	Sal	Sab	Sch	Sdo	Sla	Smu	Sra	Sre	Sru	Sst
trans-Pinocarveol	1106	-	-	-	-	-	-	0.2	-	-	-	-
cis-p-2-Menthen-1-ol	1110	-	-	-	-	-	-	-	-	-	-	tr
allo-Ocimene	1110	-	-	-	-	0.8	-	-	-	-	-	-
trans-Verbenol	1114	-	-	-	-	-	-	0.1	-	-	-	-
Pinocarvone	1121	-	-	-	-	-	-	0.1	-	-	-	-
Nerol oxide	1127	-	-	-	-	0.1	-	-	-	-	-	-
δ-Terpineol	1134	tr	0.2	-	0.2	-	0.5	-	-	-	-	-
Borneol	1134	-	-	tr	-	-	-	0.6	-	1.0	1.2	2.2
Terpinen-4-ol	1148	0.5	0.6	-	0.4	0.8	1.0	0.9	7.1	-	0.7	0.8
α-Terpineol	1159	0.7	0.8	2.7	0.5	6.2	1.3	0.7	0.7	tr	0.6	0.8
Myrtenol	1168	-	-	1.5	-	-	-	0.2	-	tr	0.8	3.5
trans-Carveol	1189	tr	tr	tr	tr	-	0.1	-	-	tr	-	-
Cuminaldehyde	1200	-	-	tr	-	-	-	-	-	tr	-	-
Carvone	1206	0.3	tr	tr	0.1	-	tr	-	-	-	-	-
Citronellol	1210	-	-	-	-	0.4	-	-	-	-	-	-
Geraniol	**1236**	**-**	**-**	**-**	**-**	**19.6**	**-**	**-**	**-**	**-**	**-**	**-**
Linalyl acetate	**1245**	**-**	**-**	**-**	**-**	**19.6**	**-**	**-**	**-**	**-**	**-**	**-**
Cumin alcohol	1260	-	-	-	-	-	-	-	-	tr	-	-
Bornyl acetate	1265	-	-	tr	-	-	-	1.8	-	1.3	0.1	-
Neryl acetate	1275	-	-	-	-	4.0	-	-	-	-	-	-
cis-Jasmone	1372	tr	tr	-	tr	-	tr	0.1	0.4	-	0.1	-
Geranyl acetate	1370	-	-	-	-	2.3	-	-	-	-	-	-
α-Gurjunene	1400	tr	1.1	-	0.6	tr	0.2	-	-	1.6	-	-
Isocaryophyllene	1406	-	-	-	-	-	-	tr	8.6	-	0.2	-
β-Caryophyllene	**1414**	**1.9**	**3.4**	**2.1**	**1.7**	**0.3**	**5.7**	**2.2**	**tr**	**8.0**	**10.5**	**2.1**
β-Gurjunene*	1426	0.7	0.3	-	0.2	-	0.1	-	-	1.4	-	-
Aromadendrene	1428	0.9	2.7	2.0	2.1	-	0.8	0.4	tr	-	tr	-
α-*trans*-Bergamotene	1434	-	-	-	-	-	-	0.3	tr	-	0.7	1.1
Eudesmadiene*	1435	0.8	tr	-	0.4	-	tr	-	-	-	-	tr
α-Humulene	1447	1.2	1.9	tr	1.0	0.2	4.7	0.2	1.6	2.8	2.3	-
trans-β-Farnesene	1455	-	-	-	-	0.2	-	0.1	-	-	0.4	0.5
allo-Aromadendrene	1456	tr	1.3	tr	1.0	-	2.4	-	-	0.9	-	-
Geranyl-2-butylether	1466	-	-	-	-	1.8	-	-	-	-	-	-
ar-Curcumene	1475	-	-	-	-	-	-	-	-	-	0.1	-
β-Selinene	1476	-	-	-	-	-	-	tr	-	-	0.1	-
Viridiflorene	1487	0.2	1.3	-	0.7	0.8	0.2	-	-	1.8	-	-
UI-C	1492	-	-	-	-	1.4	-	-	-	-	-	-
UI-A	1494	-	-	-	-	3.5	-	-	-	-	-	-
β-Bisabolene	1495	-	-	-	-	-	-	0.1	-	-	0.6	3.0

Components	RI	Sac	Sal	Sab	Sch	Sdo	Sla	Smu	Sra	Sre	Sru	Sst
γ-Cadinene	1500	1.5	4.7	-	0.6	-	2.5	-	-	0.8	-	-
trans-Calamenene	1505	0.3	0.9	-	tr	-	tr	-	-	-	-	-
β-Sesquiphellandrene	1508	-	-	-	-	-	-	-	-	-	0.2	-
trans-Nerolidol	1549	1.4	3.5	2.1	1.6	-	2.2	1.8	-	-	0.7	-
β-Caryophyllene alcohol	1550	1.4	2.0	-	1.6	-	2.2	-	-	-	-	-
UI-D	1550	-	-	-	-	2.8	-	-	-	-	-	-
Spathulenol	**1551**	**29.1**	**2.0**	**2.0**	**tr**	**1.5**	**18.3**	**-**	**7.7**	**2.6**	**tr**	**-**
β-Caryophyllene oxide	**1561**	**14.6**	**1.9**	**5.6**	**0.6**	**-**	**14.3**	**0.6**	**22.6**	**4.9**	**1.3**	**-**
Globulol	1566	tr	0.8	2.2	0.5	-	0.6	-	-	-	-	-
Viridiflorol	**1569**	**0.0**	**3.3**	**24.5**	**9.3**	**-**	**0.9**	**1.9**	**1.3**	**5.3**	**tr**	**-**
UI-B	1569	-	-	-	-	2.8	-	-	-	-	-	-
Humulene epoxide*	**1579**	**-**	**-**	**-**	**-**	**-**	**-**	**0.4**	**12.6**	**-**	**0.8**	**-**
Ledol	1580	6.5	0.0	6.6	1.4	-	5.2	-	-	4.6	-	-
epi-Cubenol	1600	0.0	1.8	-	0.4	-	0.6	-	-	-	-	-
T- Cadinol	1616	3.0	1.9	2.0	2.3	-	1.9	-	-	1.1	-	tr
δ-Cadinol	1618	-	-	-	-	0.6	-	-	1.8	1.1	tr	-
β-Eudesmol	1620	0.7	1.7	tr	0.1	-	1.3	-	-	-	-	-
Cubenol	1624	-	-	-	-	-	-	-	1.4	-	tr	-
α-Cadinol	1626	-	-	tr	-	-	-	-	-	3.1	-	-
α-Eudesmol	1634	2.0	tr	-	1.1	-	0.4	-	-	-	-	-
α-Bisabolol	**1656**	**-**	**-**	**-**	**-**	**-**	**-**	**0.2**	**3.8**	**-**	**65.5**	**26.1**
epi-α-Bisabolol	1658	-	-	2.0	-	-	-	-	-	1.4	-	-
(Z)- Lanceol	1692	-	-	-	-	-	-	-	-	-	0.2	1.3
α-Bisabolol oxide A*	1702	-	-	-	-	-	-	0.1	1.4	-	0.8	1.0
% Identification		**78.0**	**77.5**	**80.8**	**95.8**	**78.6**	**81.1**	**97.5**	**72.9**	**94.4**	**90.0**	**88.0**
Monoterpenes hydrocarbons		8.8	35.6	13.8	25.7	2.3	9.0	53.0	0.4	32.6	0.8	41.5
Oxygen containing monoterpenes		3.0	4.5	15.9	42.8	71.8	6.2	36.2	8.7	20.4	4.6	11.4
Sesquiterpene hydrocarbons		7.5	17.6	4.1	8.3	1.5	16.6	3.3	10.2	17.3	15.1	6.7
Oxygen containing sesquiterpenes		58.7	18.9	47.0	18.9	2.1	47.9	5.0	52.6	24.1	69.3	28.4
Others		0.0	0.9	0.0	0.1	0.1	1.4	0.0	1.0	0.0	0.2	0.0
Monoterpenes		11.8	40.1	29.7	68.5	74.1	15.2	89.2	9.1	53.0	5.4	52.9
Sesquiterpenes		66.2	36.5	51.1	27.2	3.6	64.5	8.3	62.8	41.4	84.4	35.1

RI: Relative retention indices calculated against *n*-alkanes. Sac: *Salvia africana-caerulea*; Sal: *S. africana-lutea*; Sab: *S. albicaulis*; Sch: *S. chamelaeagnea*; Sdo: *S. dolomitica*; Smu: *S. muirii*; Sla: *S. lanceolata*; Sra: *S. radula*; Sre: *S. repens*; Sru: *S. runcinata*; Sst: *S. stenophylla.* UI-A: unidentified compound A; UI-B: unidentified compound B; UI-C: unidentified compound C; UI-D: unidentified compound D; - : not present, tr: trace (< 0.05 %); *: determined by mass spectra only.

The components of essential oils identified in high amounts (> 10%) include α-pinene, 1,8-cineole, linalool, limonene, myrcene, β-caryophyllene, spathulenol, β-caryophyllene oxide, viridiflorol, δ-3-carene and α-bisabolol. The components identified in all 11 species include β-pinene, *p*-cymene, α-terpineol and β-caryophyllene. The essential oil composition of each species is described briefly below.

***Salvia africana-caerulea*:** Forty-three components representing 78.0% of the total oil were identified. The oil was dominated by oxygen-containing sesquiterpenes (58.7%). The percentage of monoterpenes, both monoterpene hydrocarbons and oxygen-containing monoterpenes, represent only 11.8% of identified oil components (Table 4.1). Major components of this oil include spathulenol (29.1%), β-caryophyllene oxide (14.6%) followed by ledol (6.5%) and α-pinene (4.1%). These four compounds represent up to 70% of the total oil (Figure 4.3).

***Salvia africana-lutea*:** Forty-three components representing 77.5% of the total oil composition were characterized. Monoterpene hydrocarbons (35.6%) and sesquiterpenes (36.5%), both oxygen-containing sesquiterpenes and sesquiterpene hydrocarbons, are the major identified groups of components with myrcene (11.5%), *p*-cymene (7.6%), α-pinene (6.0%) and *cis*-β-ocimene (5.4%) being the major components. They constitute about 39% of the total oil identified in this species (Figure 4.3).

***Salvia albicaulis*:** Thirty-eight components were identified in *S. albicaulis* essential oil and account for 80.8% of the total oil. This oil was characterized by the predominance of oxygen-containing sesquiterpenes (47.0%) compared to monoterpenes (29.7%) (Table 4.1). Viridiflorol (24.5%), 1,8-cineole (9.4%) and limonene (9.4%) were the most prominent components followed by ledol (6.6%) and β-caryophyllene oxide (5.6%). All these compounds represent 69% of the total oil (Figure 4.3).

***Salvia chamelaeagnea*:** Forty-three components were identified in this species representing 95.8% of the total oil composition. Oxygen-containing monoterpenes and oxygen-containing sesquiterpenes represent 42.8% and 18.9% of the identified groups of components, respectively. This oil is characterized by high levels of 1,8-cineole (40.5%) followed by α-pinene (10.4%), limonene (9.7%) and viridiflorol (9.3%) (Table 4.1). These four compounds constitute 73% of the total oil of *S. chamelaeagnea* (Figure 4.3).

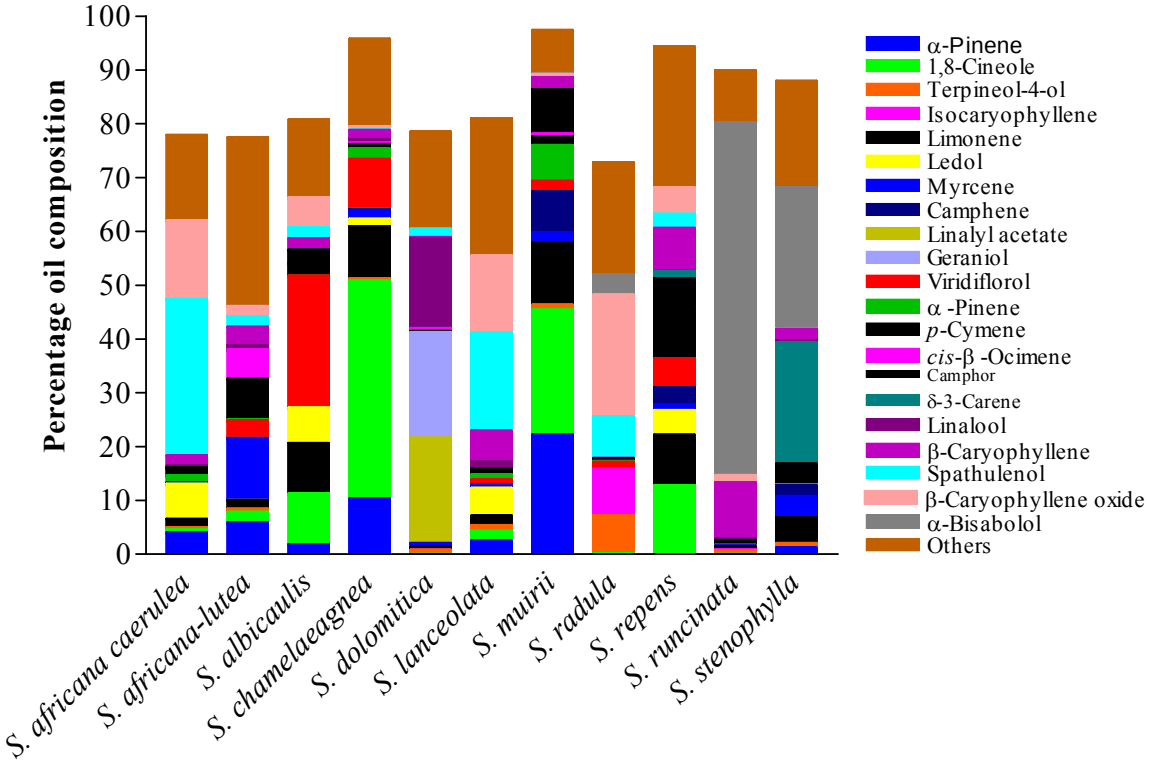


Figure 4.3 Variation in relative amounts of common and major components of the essential oils of eleven indigenous *Salvia* species: Sac: *Salvia africana-caerulea*; Sal: *S. africana-lutea*; Sab: *S. albicaulis*; Sch: *S. chamelaeagnea*; Sdo: *S. dolomitica*; Smu: *S. muirii*; Sla: *S. lanceolata*; Sra: *S. radula*; Sre: *S. repens*; Sru: *S. runcinata*; Sst: *S. stenophylla.*

***Salvia dolomitica*:** Thirty-six components representing 78.6% of the total oil were identified in *S. dolomitica*. The oil of this species is characterized by a high level of oxygen-containing monoterpenes (71.8 %), while the sesquiterpenes represent only 3.6% of the identified components. The major components identified include geraniol (19.6%), linalyl acetate (19.6%), linalool (16.6%) and α-terpineol (6.2%) (Table 4.1). They represent 79% of the compounds identified in *S. dolomitica* oil (Figure 4.3). In *S. dolomitica*, four compounds amounting 11% of the total oil were detected at RIs 1494, 1569, 1492 and 1550 but remained unidentified (UI) and were assigned as UA-A, UI-B, UI-C and UI-D, respectively. The mass spectra of these four compounds previously detected in species belonging to the family Umbelliferae are shown in Figure 4.4.

***Salvia lanceolata*:** Forty-three components accounting for 81.1% of the total oil were identified. The oil had a predominance of oxygen-containing sesquiterpenes (47.9%),

while the monoterpenes represented only 15.2% of the identified components. Major components were spathulenol (18.3%), β-caryophyllene oxide (14.3%) followed by β-caryophyllene (5.7%) and ledol (5.2%) (Table 4.1). The four compounds constitute 54% of the total oil of this species (Figure 4.3).

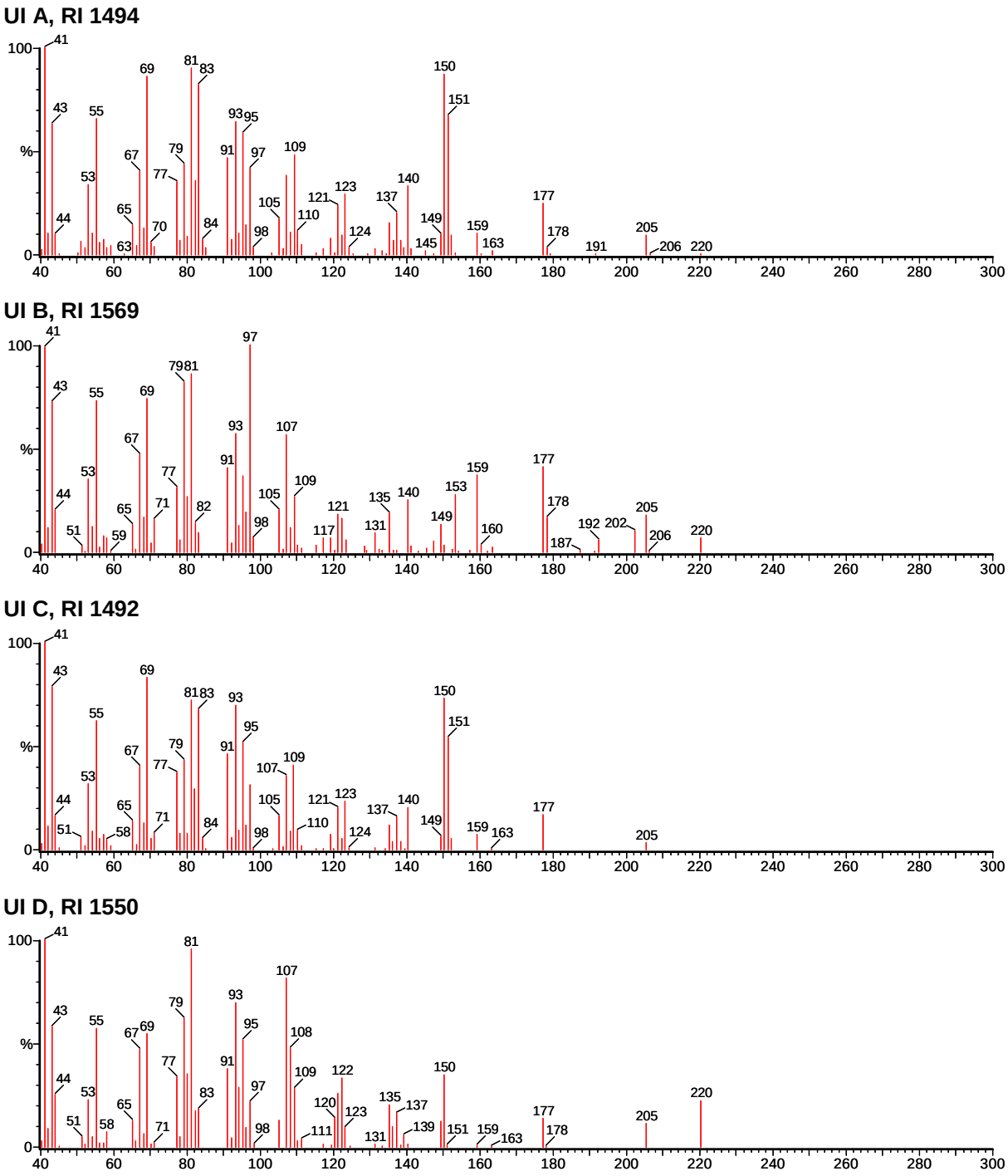


Figure 4.4 Mass spectra of the unidentified compounds [RIs 1494 (UI-A), 1569 (UI-B), 1492 (UI-C) and 1550 (UI-D)] present in *Salvia dolomitica* oil.

***Salvia muirii*:** Thirty-nine components representing 97.5% of the oil were identified and this oil was dominated by the monoterpene hydrocarbons (53.0%) and oxygen-containing monoterpenes (36.2%) as the major classes of components, while sesquiterpenes represented only 8.3% of the identified components (Table 4.1). The major components of this oil include 1,8-cineole (23.2%), α-pinene (22.3%) followed by limonene (11.6%), camphor (8.6%), camphene (7.6%) and β-pinene (6.7%). They represent 82% of the identified compounds in *S. muirii* oil (Figure 4.3).

***Salvia radula*:** As shown in Table 4.1, GC and GC-MS analysis of the oil resulted in the identification of only 22 components representing 72.9% of the oil. The major class of components was the oxygen-containing sesquiterpenes (52.6%), while sesquiterpene hydrocarbons and monoterpenes represented only 10.2% and 9.1% of the identified components, respectively (Table 4.1). β-Caryophyllene oxide (22.6%) and humulene epoxide (12.6%) were the most prominent components followed by isocaryophyllene (8.6%), spathulenol (7.7%) and terpineol-4-ol (7.1%). They represent 80% of the total oil (Figure 4.3).

***Salvia repens*:** Analysis of the essential oil resulted in the characterization of thirty-six components representing 94.4% of the oil. Monoterpene hydrocarbons (32.6%) and oxygen-containing sesquiterpenes (24.1%) were the major structural groups of compounds in this oil. The oil has a predominance of 1,8-cineole (12.8%), *p*-cymene (9.5%) and limonene (9.4%), followed by β-caryophyllene (8.0 %), camphor (5.3%) and viridiflorol (5.3%) (Table 4.1). They amount to 53% of the total oil (Figure 4.3).

Salvia runcinata: Forty-two components representing 90.0% of the total oil were identified and the major structural class of compound was oxygen-containing sesquiterpenes (69.3%), while monoterpenes represented only 5.4% of the identified components (Table 4.1). This oil was dominated by α-bisabolol (65.5%) and β-caryophyllene (10.5%). These two compounds represent 84% of *S. runcinata* oil (Figure 4.3).

***Salvia stenophylla*:** The results of the GC and GC-MS showed that 36 components representing 88.0 % of the oil could be identified. This oil was dominated by monoterpene hydrocarbons (41.5%) and oxygen-containing sesquiterpenes (28.4%) with α-bisabolol

(26.1%) and δ-3-carene (22.6%) being the most prominent components followed by limonene (4.8%) (Table 4.3). These compounds contribute to 61% of the total oil composition (Figure 4.3).

4.4.3 Cluster analysis

The cluster analysis was constructed using the 93 essential oil components identified in the 11 *Salvia* species and UI-A, UI-B, UI-C and UI-D and interpreted according to Pestana and Gageiro (2000). Two major clusters (A and B) could be observed (Figure 4.5). One cluster consists of *S. dolomitica* alone, while the remaining species constitute another cluster. A very high correlation was observed between the essential oil of *S. africana-caerulea* and *S. lanceolata* ($S_{corr} \approx 0.92$) (Figure 4.5). This strong correlation is based on the abundance of spathulenol and β-caryophyllene oxide present in the two plants (Table 4.1).

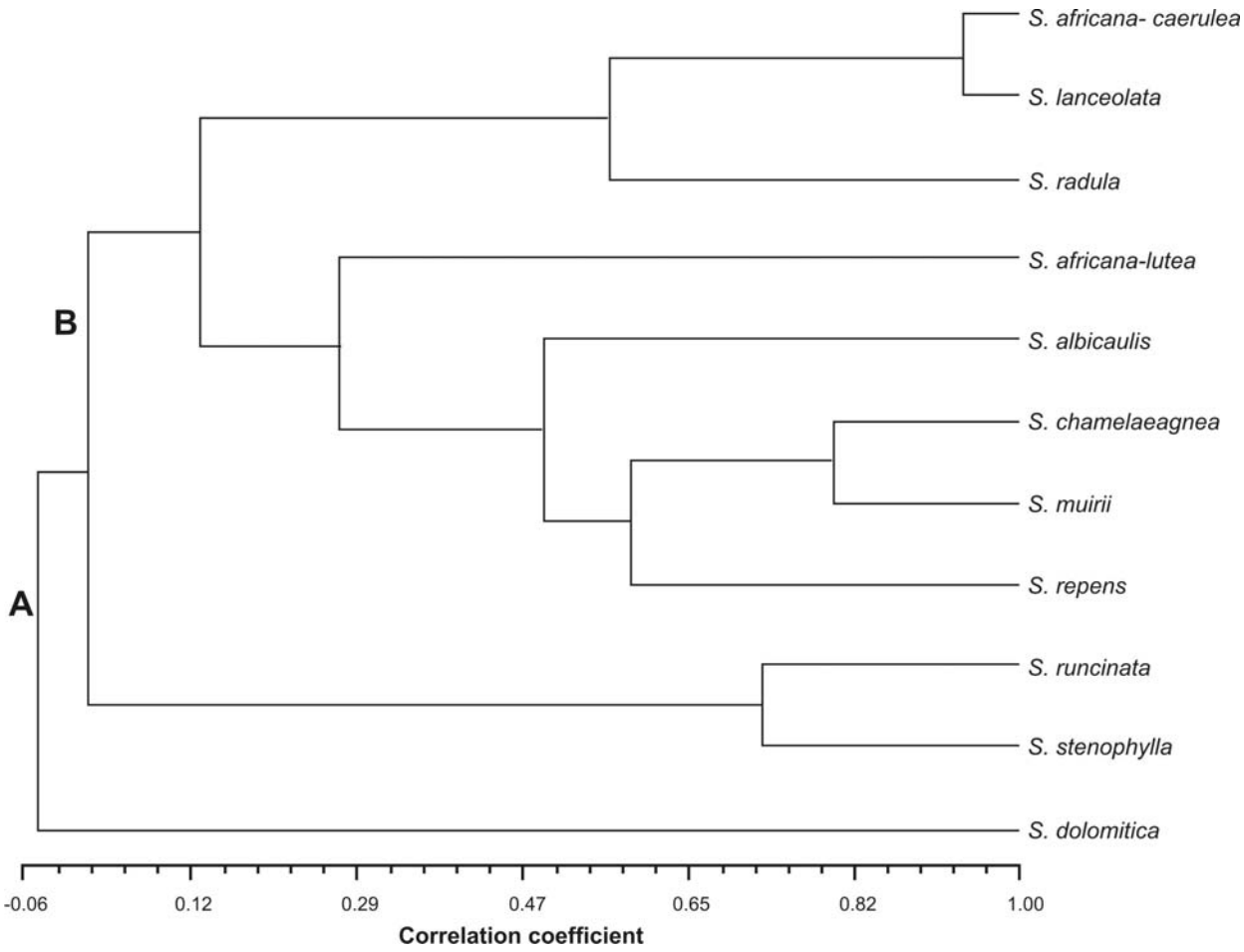


Figure 4.5 Cluster analysis of eleven *Salvia* species constructed using the essential oil composition data.

A high correlation was obtained between *S. chamelaeagnea* and *S. muirii* ($S_{corr} \approx 0.77$) (Figure 4.5) due to the presence of α-pinene, 1,8-cineole and limonene in relatively high amounts. A high correlation was also observed between *S. runcinata* and *S. stenophylla* ($S_{corr} \approx 0.71$) with both containing high amounts of α-bisabolol (Table 4.1). Moderate correlation was observed between *S. albicaulis*, *S. chamelaeagnea*, *S. muirii* and *S. repens* and also between *S. radula*, *S. africana-caerulea* and *S. lanceolata* (Figure 4.5). *S. africana-lutea* and *S. dolomitica* have a low correlation with the nine other species as indicated by the low correlation coefficient ($S_{corr} < 0.30$).

4.5 Discussion

This investigation showed variability in yield of essential oils of the species studied with *S. muirii* having the highest yield and *S. radula* the lowest. The yield obtained in this study was relatively low compared to those reported in literature for other *Salvia* species. Bellomaria *et al*. (1992) reported the yield of *S. pomifera*, *S. willeana* and *S. fruticosa* to be 1.3, 2.8 and 5.3%, respectively. Pitarevic *et al*. (1984) found that the yield of *S. officinalis* collected at various seasons throughout the year varied from 1.8 to 3.1%. The difference obtained in yield may be explained by the fact that in the current study, the yield was expressed based on wet biomass, while in other studies, the dry biomass was used. Furthermore, difference in yield varies with soil types, plant part used, climate, season and stage of plant development (Putievsky *et al*., 1986; Bellomaria *et al*., 1992). The relatively high yield obtained in *S. muirii* and *S. chamelaeagnea* may be attributed to high density of glandular trichomes as it has been reported that these trichomes are responsible for the secretion of the essential oils (Werker *et al*., 1985; Karousou *et al*., 1992).

The essential oils are qualitatively and quantitatively heterogeneous (Figure 4.4). Constituents of essential oils such as α-pinene, 1,8-cineole, linalool, limonene, myrcene, β-caryophyllene, spathulenol, β-caryophyllene oxide, viridiflorol, δ-3-carene and α-bisabolol were identified in greater amounts (Table 4.1). Components identified in all the 11 species include β-pinene, myrcene, *p*-cymene, α-terpineol and β-caryophyllene. Many components identified in the current study have been previously identified in non-indigenous *Salvia* species and these include 1,8-cineole, myrcene, β-caryophyllene, borneol, camphene, limonene and spathulenol (Pitarevic *et al*., 1984; Bellomaria *et al*., 1992; Ahmed *et al*.,

1994; Carta *et al*., 1996; Foray *et al*., 1999; Kaya *et al*., 2003). However, in this study, high levels of geraniol and linalyl acetate were detected in *S. dolomitica* (Table 4.1) in this study, which have rarely been identified in exotic *Salvia* species (Bellomaria *et al*., 1992; Farhat *et al*., 2001). It is important to note that α-thujene and β-thujene, which are major components of the essential oil of *S. offinalis* (Pitarevic *et al*., 1984), were detected in relatively low amounts (Table 4.1). Many factors may explain variation in essential oil composition between different species. In addition to the genetic make up of each species and factors listed above which affect the essential oil yield, the mode of harvest and isolation procedure may also influence the essential oil composition (Pitarevic *et al*., 1984; Bellomaria *et al*., 1992). Ahmed *et al*. (1994) investigated the essential oil of *S. hispanica* L. collected from three geographical areas and found major variations in their essential oil composition. Brunke and Hammerschmidt (1985) analysed the essential oil of *S. stenophylla* and found that δ-carene (20.0%) and α-bisabolol (29.8%) were the dominant components. In the current study, the two components represent 22.6 and 26.1%, respectively (Table 4.1). Jequier *et al*. (1980) found that oxygen-containing monoterpenes represent 5% of the oil and the sesquiterpene hydrocarbons 35% in *S. stenophylla*. The amount of oxygen-containing monoterpenes and sesquiterpene hydrocarbons is this investigation was 11.4% and 6.7%, respectively (Table 4.1).

There are many components that were identified in one plant but were absent in others (Table 4.1 and Figure 4.4) and this confirms the heterogeneity of the oils investigated. Based on Cabo *et al*. (1987) classification, the essential oils of *Salvia* species can be classified into three groups: Group I is composed of species with a predominance of α-thujene, β-thujene and 1,8-cineole; group II is composed of essential oils rich in linalool and linalyl acetate; and group III comprises species rich in 1,8-cineole and camphor. The oils examined in this study do not fall into any of the groups as described by Cabo *et al*. (1987). Apparently, these findings display new groups (Table 4.1). *S. runcinata* may be a potential natural source α-bisabolol since it constitutes more than 65% of the total oil. Components of the essential oil present in only one species and absent in others with an amount greater than 1% include geraniol (19.6%), linalyl acetate (19.6%), neryl acetate (4.0%), geranyl-2-butylether (1.8%), cubenol (1.4%) and unidentified components (UI-A, UI-B, UI-C and UI-D) with 1.4%, 2.8%, 1.4% and 2.8%, respectivel (Table 4.1). Eight components, including the unidentified (UI) UI-A, UI-B, UI-C and UI-D, representing 55.5% of the total oil, were found only in *S. dolomitica* and were absent in other species.

The presence of these components can explain the very low correlation coefficient found between the essential oil of *S. dolomitica* and the rest of the species (Figure 4.4). The unidentified components of the oils namely UI-A, UI-B, UI-C and UI-D were previously detected in species of the family Umbelliferae (Figueiredo, *pers comm*), but have not yet been characterised. Their presence in *S. dolomitica* differentiates this species from others indigenous *Salvia* species to date. The oil composition of *S. africana-caerulea* resembles that of *S. lanceolata* (Table 4.1) and this is supported by the cluster analysis (Figure 4.5). Codd (1985) placed *S. africana-caerulea*, *S. africana-lutea*, *S. chamelaeagnea* and *S. lanceolata* within the same group based on morphological characters and SEM observations (Chapter 3).

The essential oil of *S. chamelaeagnea* in this study resembles that of *S. muirii* as shown by the high correlation coefficient found between the two species (Figure 4.5). On the other hand, low similarity was found in essential oil composition of *S. africana-lutea* and other species within the complex. In the current study a very high correlation was found between S. *africana-caerulea* and *S. lanceolata* and provides chemotaxonomic support for the affinity as described by Codd (1985). Similarly, *S. repens*, *S. runcinata* and *S. stenophylla* were also placed in the same group and a high correlation was found between the essential oil of *S. runcinata* and *S. stenophylla* (Figure 4.5). The essential oil composition data has provided additional information in order to better understand the the chemistry of the investigated species which will in turn help to understand the biological activities of the essential oils studied in the next Chapters.

4.6 Conclusions

- The essential oil yield varied between species, with *S. muirii* giving the highest yield and *S. radula* the lowest yield.
- Quantitative and qualitative variations were observed between the essential oils of the investigated species.
- *S. runcinata* is the only species where one compound (α-bisabolol) represents more than 65% of the total oil.
- A very high correlation was found between the essential oil of *S. africana-caerulea* and *S. lanceolata* due to their high amount of spathulenol and β-caryophyllene oxide.

- The correlation between the essential oil of *S. dolomitica* and the rest of the species was very low due to the presence of geraniol, linalyl acetate and four other unidentified components.
- The essential oil data provides chemotaxonomical confirmation previously suggested relationships between taxa based on morphological evidence alone.

Chapter 5: HPLC-UV-MS Analysis of the Solvent Extracts

Abstract

Salvia species are rich in phenolic compounds which are partly responsible for the various biological activities. High performance liquid chromatography was used to detect caffeic acid, carnosic acid, kaempferol, oleanolic acid, rosmarinic acid and ursolic acid in 17 solvent extracts. Compounds isolated from *S. chamelaeagnea* (7-*O*-methylepirosmanol and carnosol) and *S. radula* (betulafolientriol oxide and salvigenin) were also included to verify their presence in other *Salvia* species. Betulafolientriol oxide was detected in all the investigated species. Rosmarinic acid, carnosic acid, carnosol and oleanolic acid/ursolic acid were detected in relatively high levels. The level of rosmarinic acid (percentage area) ranged from 3.4 (*S. radula*) to 52.8% (*S. muirii*) and was absent in *S. verbenaca*. *S. garipensis* and *S. radula* were the only species which lacked oleanolic acid and ursolic acid. Kaempferol, a compound commonly found in exotic *Salvia* species was not detected in any of the species studied. The solvent extracts also contained other flavonoids (e.g. flavones).

5.1 Introduction

Salvia is an important genus widely cultivated and used in flavouring and is a popular ingredient folk medicines. They are used in traditional medicine in many parts of the world for the treatment of various diseases (Liu *et al*., 1992). The genus has attracted great interest, and has been the subject of numerous phytochemical studies (Lu and Foo, 2002). It is a rich source of polyphenols, with an excess of 160 polyphenols having been identified, of which many are unique to the genus (Lu and Foo, 2002).

Many *Salvia* species have received interest due to their ability to accumulate high levels of active compounds, notably flavonoids (Tanaka *et al*., 1989; Ulubelen *et al*., 2001). These classes of compounds are known to exhibit a range of biological activities including; anticancer, antibacterial, anti-oxidant and anti-inflammatory properties (Ulubelen *et al*., 2001, Cuvelier *et al*., 1994; Lu and Foo, 2002; Kobayashi *et al*., 2002). Many Lamiaceae extracts are of commercial interest to the food industry as a source of natural anti-oxidants (Thorsen and Hildebrandt, 2003). The quality of anti-oxidant and the price of commercially available species, such as *Rosmarinus officinalis* extracts, are highly correlated with the total phenolic compounds, for example the levels of carnosic acid, rosmarinic acid and caffeic acid (Thorsen and Hildebrandt, 2003). A large number of polyphenolic compounds are constructed from the caffeic acid building block via a variety of condensation reactions (Lu and Foo, 2002). As *Salvia* species are also rich in phenolic compounds, accurate identification is of great importance for commercial development. The identification of these compounds will also help to understand the pharmacological properties of species studied.

High performance liquid chromatography (HPLC) of phenolic and triterpenoid compounds is a widely used methodology designed to separate, quantify, identify and analyse components in a chemical mixture (Areias *et al*., 2001).

The objectives of this study were to:

(i) determine the presence of some phenolic and triterpenoid compounds, previously identified in exotic *Salvia* species (caffeic acid, carnosic acid, kaempferol, oleanolic acid, rosmarinic acid and ursolic acid) in the solvent extracts of indigenous *Salvia* species,

(ii) confirm the presence of compounds isolated from *S. chamelaeagnea* (7-*O*-methylepirosmanol and carnosol) and *S. radula* (betulafolientriol oxide and salvigenin) in the other species, and

(iii) determine among these compounds those which are the most common in indigenous *Salvia* species.

5.2 Materials and methods

5.2.1 Chemicals, reagents and standards

Analytical grade acetonitrile, methanol and chloroform were purchased from Rochelle Chemicals, while standards of caffeic acid, carnosic acid, kaempferol, oleanolic acid, rosmarinic acid and ursolic acid were obtained from Sigma®. Carnosol and 7-*O*-methylepirosmanol were isolated from *S. chamelaeagnea* (Chapter 9), while betulafolientriol oxide and salvigenin [5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one] were isolated from *S. radula* (Chapter 8).

5.2.2 Preparation of samples

The methanol:chloroform (1:1) extracts were prepared as described in Section 2.3.2. The 17 samples and standards for the analysis were prepared at a concentration of 50 mg/ml in methanol. They were filtered using Whatman® No 1 filter paper, stored in vials at 4 ºC until analysed.

5.2.3 High performance liquid chromatography

The detection and identification of compounds were performed using a Waters 2695 HPLC system equipped with both a 2996 photodiode array (PDA) detector and a Thermabeam electron impact (EI) mass selective detector (TMD). The TMD detector was operated in EI mode with the ionizer at 70 eV (fixed) with a gain of 10 and scanning mass range of 50-550 amu. Analysis was performed on a Phenomenex Aqua C18 column (250 x 2.1 mm, 5 µm) thermostated at 40 ºC. The flow rate of the HPLC was 0.2 ml/min and the gas flow through the nebulizer 30 l/h. The nebulizer temperature was 80 ºC, the expansion region 90 ºC and the source temperature 225 ºC. The TMD was operated in positive ion mode

with no flow splitting, thereby utilizing the full HPLC eluent at a flow rate of 0.2 ml/min. The mobile phase started with 10% acetonitrile, 90% water containing 10 mM formic acid. The solvent ratio was changed through a linear gradient to 90% acetonitrile, 10% water (with 10 mM formic acid) at 40 minutes. This ratio was maintained for 10 minutes where after the solvent ratio was changed back to the initial conditions. Standards of caffeic acid, carnosic acid, kaempferol, oleanolic acid, rosmarinic acid and ursolic acid (10 µl, 50 mg/ml) and isolated compounds (50 mg/ml) from *S. chamelaeagnea* (carnosol and 7-*O*-methyl-epi-rosmanol) and *S. radula* (betulafolientriol oxide and salvigenin) were injected separately and co-injected with samples (10 µl) in order to identify and confirm their presence in the extracts.

5.3 Data analysis

Comparison of the MS spectra of standards and isolated compounds after fragmentation of the parent ion together with retention time (Rt) and ultra-violet (UV) spectra were used to confirm the presence/absence of these compounds in the solvent extracts using the Empower® software. Flavonoids were tentatively assigned by comparing the UV absorption spectra of different flavonoids (Markham, 1982). The area (%) was calculated from the integrated peak area.

5.4 Results

5.4.1 Identification of compounds

The typical UV spectra (Figure 5.1) and the chromatographic data (Table 5.1) obtained for the standards and isolated compounds are shown below.

The HPLC analysis revealed the presence of various compounds in *Salvia* species (Table 5.2). Betulafolientriol oxide, a compound isolated from *S. radula* was the only compound identified in all 17 species at relatively low levels (Table 5.2). Rosmarinic acid, carnosic acid, carnosol and ursolic acid were present in the majority of the extracts in relatively high levels. The percentage area of rosmarinic acid ranged from 3.41 (*S. radula*) to 52.82% (*S. muirii*) (Table 5.2). *S. albicaulis* (35.23%), *S. runcinata* (38.40%) and *S. muirii* (52.82%) were particularly rich in rosmarinic acid (Table 5.2) and *S. verbenaca* was the only species

in which rosmarinic acid was not detected. Carnosol and/or carnosic acid was/were abundant in *S. aurita*, *S. chamelaeagnea*, *S. namaensis* and *S. stenophylla* (area > 20%). Salvigenin (23.76%) and oleanolic acid/ursolic acid (19.85%) were abundant in *S. disermas* (Table 5.2).

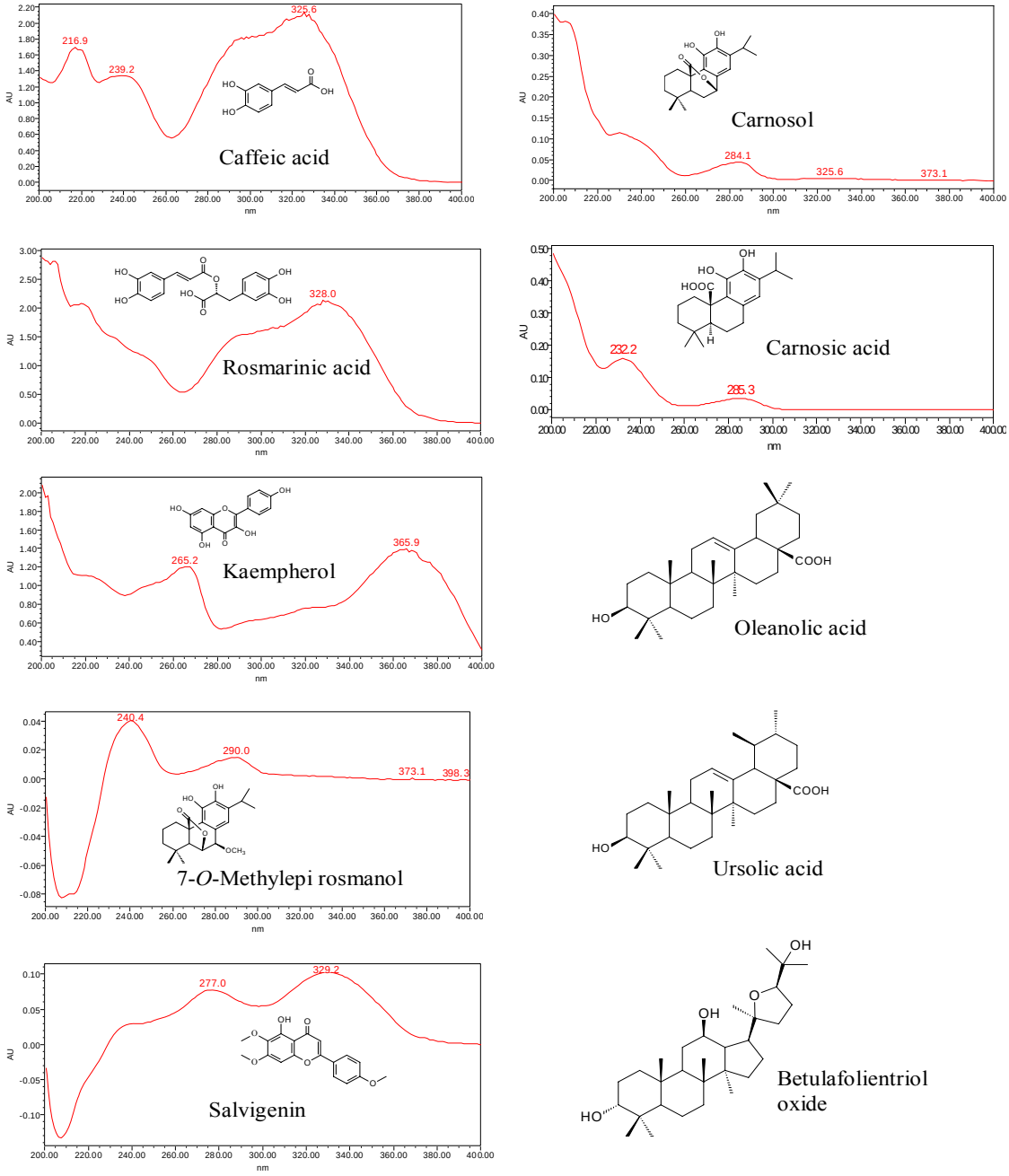


Figure 5.1 UV spectra of standards and isolated compounds from *S. radula* and *S. chamelaeagnea*. The UV spectra of ursolic acid, oleanolic acid and betulafolientriol oxide are not reported because they are not good chromophores.

Table 5.1 Chromatographic data obtained for the various standards according to their retention times.

Number	Standards/Isolated compounds	Rt (min)	Absorption maxima (λ_{max}) in nm
1	Caffeic acid	15.41	217, 239, 292, 328
2	Rosmarinic acid	23.25	204, 328
3	Kaempferol	30.38	266, 365
4	7-*O*-Methylepirosmanol	34.39	240, 290, 373, 378
5	Salvigenin	34.60	216, 277, 330
6	Carnosol	34.85	284, 323, 373
7	Carnosic acid	37.03	232, 285
8	Oleanolic acid	41.11	-
9	Ursolic acid	41.09	-
10	Betulafolientriol oxide	43.28	-

-: poor UV absorption

The only other species in which 7-*O*-methylepirosmanol was detected was *S. namaensis*. Kaempferol reported in exotic *Salvia* species was absent in all the 17 *Salvia* species investigated (Table 5.2).

5.4.2 Detection of other flavonoids in the solvent extracts

The results of the HPLC-UV analyses of the 17 species examined showed several compounds exhibiting UV spectra typical of flavonoids (Markham, 1982). The current investigation focused mostly on flavones. Approximately 70% of the plants studied contained flavones based on UV spectra only (Table 5.3). In *S. disermas*, various flavones were recorded, while a few were noted in *S. africana-caerulea*, *S. aurita*, *S. chamelaeagnea*, *S. dolomitica*, *S. garipensis*, *S. lanceolata*, *S. namaensis*, *S. radula*, *S. repens*, *S. runcinata*, *S. schlechteri* and *S. stenophylla* (Table 5.3). In five species namely; *S. africana-lutea*, *S. albicaulis*, *S. lanceolata*, *S. muirii* and *S. verbenaca*, no flavones other than those mentioned in Table 5.2 were detected (Table 5.3). The flavone at a retention time of 20.76 min was found in *S. chamelaeagnea*, *S. dolomitica* and *S. garipensis* (Table 5.3). The HPLC chromatograms of all the investigated species are presented in Figures 5.2.

Table 5.2 Area (%) and identification of standards and isolated compounds in the solvent extracts of 17 indigenous *Salvia* species.

Species	Caffeic acid (1)	Rosmarinic acid (2)	Kaemp-ferol(3)	7-*O*-Methyl-epirosmanol (4)	Salvigenin (5)	Carnosol (6)	Carnosic acid (7)	Oleanolic/ Ursolic acid (8 or 9)	Betulafolien-triol oxide (10)
S. africana-caerulea	5.29	22.90	-	-	-	-	11.55	3.21	0.92
S. africana-lutea	-	23.82	-	-	-	-	14.22	4.49	0.75
S. albicaulis	0.58	35.23	-	-	-	1.10	-	10.23	1.26
S. aurita	0.84	24.44	-	-	-	27.61	6.37	0.10	0.68
S. chamelaeagnea	0.40	26.36	-	0.87	-	22.48	27.25	0.49	0.52
S. disermas	0.25	3.62	-	-	23.76	-	-	19.85	2.06
S. dolomitica	0.75	14.05	-	-	-	-	-	0.10	4.53
S. garipensis	-	29.22	-	-	7.38	-	-	-	2.91
S. lanceolata	3.29	10.67	-	-	-	2.05	-	14.28	3.83
S. muirii	0.87	52.82	-	-	0.10	4.83	11.80	0.05	1.41
S. namaensis	0.92	15.60	-	1.19	-	30.97	20.25	0.10	1.00
S. radula	-	3.41	-	-	8.52	-	-	-	1.94
S. repens	0.47	23.66	-	-	-	13.40	13.01	0.10	2.56
S. runcinata	0.34	38.40	-	-	3.55	-	3.36	0.20	1.05
S. schlechteri	0.33	26.58	-	-	-	10.88	10.38	0.10	0.80
S. stenophylla	-	22.17	-	-	-	23.60	25.17	0.10	1.80
S. verbenaca	-	-	-	-	1.80	-	-	2.19	2.76

- : not detected

Table 5.3 Compounds exhibiting a typical flavonoid spectrum in the solvent extracts of 17 *Salvia* species.

Retention time	$UV_{\lambda max}$ (nm)	Sac	Sal	Sab	Sau	Sch	Sdi	Sdo	Sga	Sla	Smu	Sna	Sra	Sre	Sru	Ssc	Sst	Sve
13.57	238, 328				+							+		+				
14.98	242, 327						+											
15.41	236, 327														+		+	
16.54	234, 327											+					+	
17.56	242, 329													+			+	
19.60	253, 348					+		+										
20.61	252, 341						+						+					
20.73	251, 269, 339															+		
20.76	256, 345					+		+	+									
20.86	269, 335	+																
22.70	250, 341						+									+		
23.63	252, 347								+									
27.83	269, 334					+		+										
28.55	265, 341						+	+										
30.33	288, 329	+																
30.64	252, 341						+	+										
31.48	269, 329						+									+		
33.12	269, 337						+											
41.72	272, 324				+		+											
42.48	272, 325						+											
50.46	272, 326						+											

Sac: *S. africana-caerulea*, **Sal**: *S. africana-lutea*, **Aab**: *S. albicaulis*, **Sau**: *S. aurita*, **Sch**: *S. chamelaeagnea*, **Sdi**: *S. disermas*, **Sdo**: *S. dolomitica*, **Sga**: *S. garipensis*, **Sla**: *S. lanceolata*, **Smu**: *S. muirii*, **Sna**: S. *namaensis*, **Sra**: *S. radula*, **Sre**: *S. repens*, **Sru**: *S. runcinata*, **Ssc**: *S. schlechteri*, **Sst**: *S. stenophylla*, **Sve**: *S. verbenaca*.

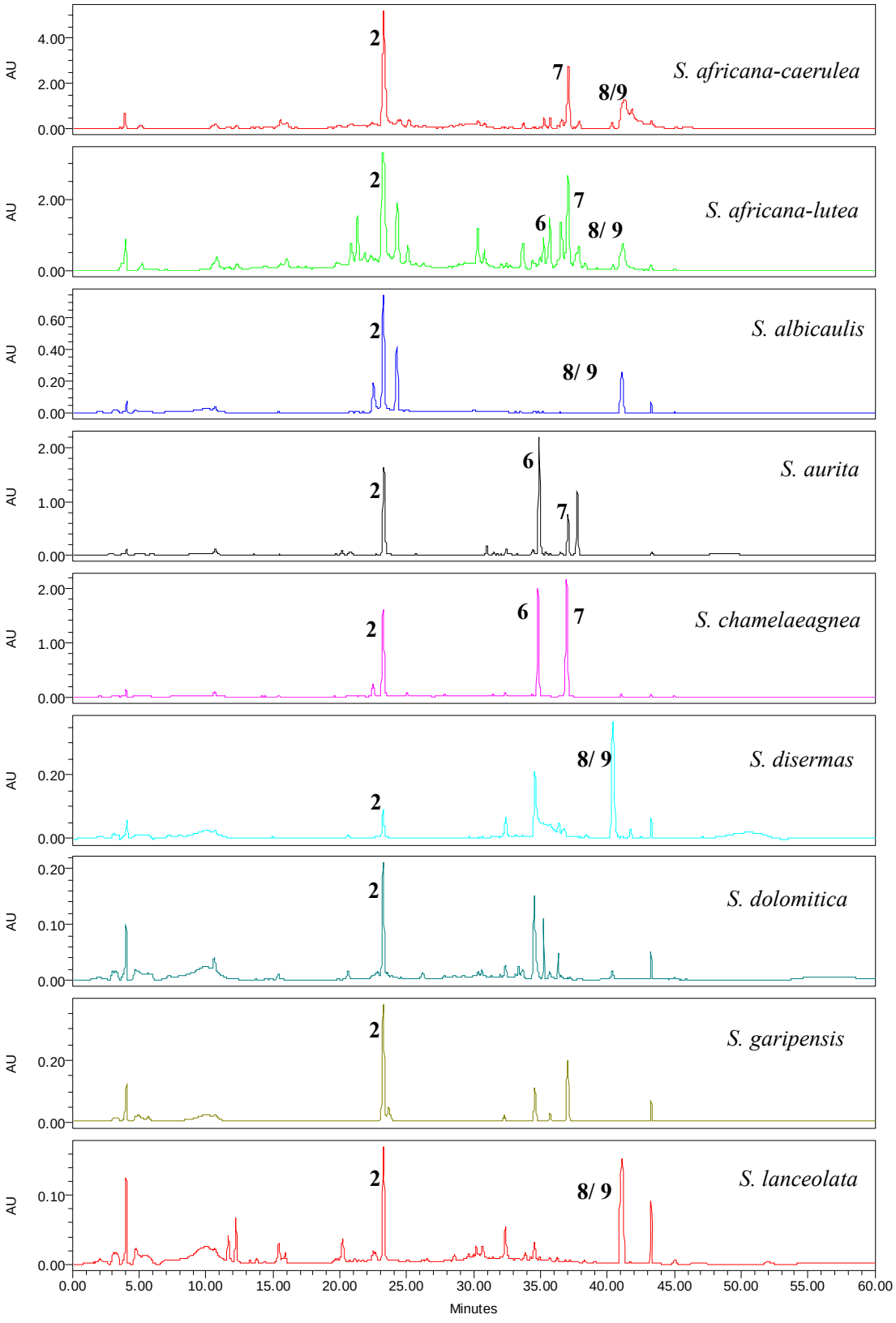


Figure 5.2 HPLC chromatograms of South African *Salvia* species. Rosmarinic acid (**2**), carnosol (**6**), carnosic acid (**7**), oleanolic acid (**8**) and ursolic acid (**9**).

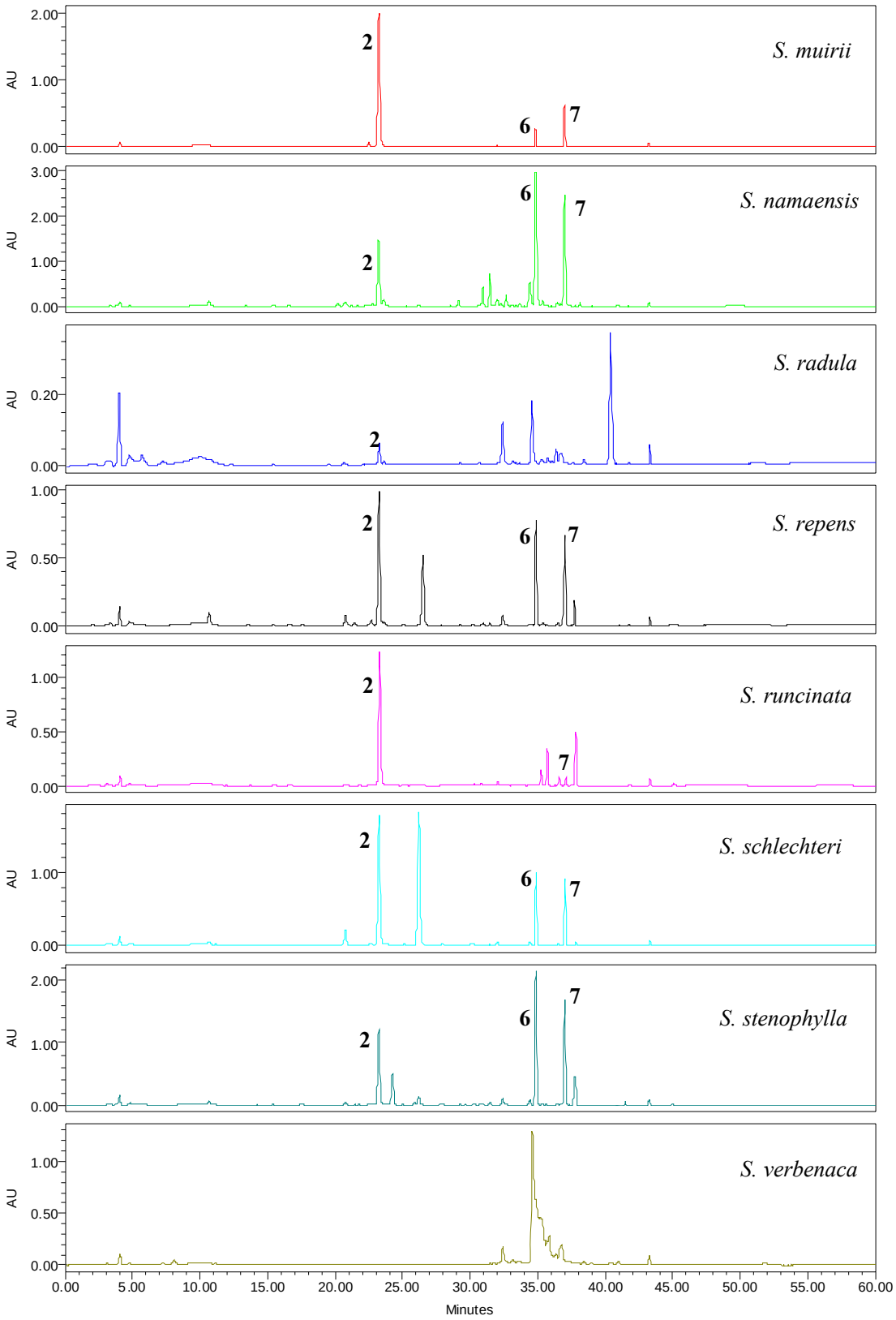


Figure 5.2 Contd. HPLC chromatograms of South African *Salvia* species. Rosmarinic acid (**2**); carnosol (**6**) and carnosic acid (**7**).

5.5 Discussion

Phenolic compounds are widely distributed in *Salvia* species (Adzet *et al*., 1987; Ulubelen and Tuzlaci, 1990) and the flavonoids are mostly present as flavones, flavonols and their glycosides (Lu and Foo, 2002). Flavones were identified in 12 species (Table 5.3), while in five species namely; *S. africana-lutea*, *S. albicaulis*, *S. lanceolata*, *S. muirii* and *S. verbenaca*, no flavones were detected (Table 5.3).

The majority of flavonoids found in exotic *Salvia* species are flavones of the apigenin and luteolin type and their corresponding hydroxylated derivatives, while the flavonols are mostly those of kaempferol and quercetin (Lu and Foo, 2002). However, kaempferol was not detected in any of the investigated species (Table 5.2).

The majority of the phenolic acids in *Salvia* species are exclusively those of caffeic acid derivatives. Caffeic acid plays a central role in the biochemistry of Lamiaceae and occurs predominantly in the dimer form as rosmarinic acid (Gerhardt and Schroeter, 1983). In many *Salvia* species, caffeic acid is the building block of a variety of plant metabolites, ranging from the simple monomers to multiple condensation products that give rise to a variety of oligomers (Lu and Foo, 2002). The trimers and tetramers are also interesting from a therapeutic point of view as they have demonstrated various biological activities such as anti-oxidant, antimicrobial and anticancer activities (Lu and Foo, 2002). The monomers that are frequently present in *Salvia* species are represented by caffeic acid (Qian and Li, 1992) and other monomeric derivatives including ferulic acid (Cuvelier *et al*., 1996) and isoferulic acid (Ai and Li, 1992). The caffeic acid was present in 70% of the solvent extracts investigated (Table 5.2). Caffeic acid was also identified in a dimer form (rosmarinic acid) in 94% of the species investigated.

Before the chemical structure of rosmarinic acid was elucidated, rosmarinic acid and similar compounds have been known as “Labiatengerbstoffe”, a type of tannin known from species of the Lamiaceae (Petersen and Simmonds, 2003). Rosmarinic acid occurs throughout the Boraginaceae whereas within the Lamiaceae it is restricted to the sub-family Nepetoideae (Janicsák *et al*., 1999). Rosmarinic acid is the most abundant caffeic dimer in *Salvia* species (Ai and Li, 1988; Cuvelier *et al*., 1994; Lu and Foo, 1999) and has been reported to be the major phenolic compound of *Salvia* samples (Cuvelier *et al*., 1996).

This was confirmed by the results obtained in this study. Rosmarinic acid was detected in nerly all the species srudied and *S. verbenaca* was the only species lacking this compound (Table 5.2). *S. albicaulis* (35.23%), *S. runcinata* (38.40%) and *S. muirii* (52.82%) contained high levels of rosmarinic acid (Table 5.2). Phenolic and triterpenoid compounds reported here have certainly contributed to various biological activities of the solvent extracts reported in the next Chapters. Several studies have reported on the detection and quantification of rosmarinic acid in various *Salvia* preparations (Janicsák and Máthé, 1997, Yaun *et al*., 1998; Janicsák *et al*., 1999; Areias *et al*., 2000). The variation in levels of caffeic acid and rosmarinic acid in the same plant has been shown. Janicsák *et al*. (1999) demonstrated that concentrations of caffeic acid were always much lower than those of rosmarinic acid. In all the species studied where the two compounds were detected together, the same pattern was observed (Table 5.2). All the compounds detected in southern African species investigated have previously been identified in exotic *Salvia* species (Cuvelier *et al*., 1996; Baricevic *et al*., 2001; Liu, 2005). As phenolic and triterpenoid compounds are known to exhibit a range of biological activities, this prompted an investigation on biological activities of the solvent extracts in the subsequent Chapters.

5.6 Conclusions

- *Salvia* species are a rich source of polyphenolic flavonoids and phenolic acids, notably rosmarinic acid, carnosic acid, caffeic acid, carnosol and ursolic acid.
- Betulafolientriol oxide, a compound isolated from *S. radula* was detected in low amount in all the species studied and if found in species at various locations, it may be used as taxonomic marker for indigenous species.
- High level of rosmarinic acid was recorded in the majority of the species studied particularly in *S. muirii* and *S. runcinata* while it was absent in *S. verbenaca*.
- *Salvia disermas* has high levels of salvigenin and oleanolic/ursolic acid.
- Kaempferol, a compound commonly detected in exotic *Salvia* species was absent in all the species studied.

Chapter 6: Anti-Oxidant Activity and Total Phenolic Content

Abstract

The anti-oxidant activity of 17 solvent extracts and 11 essential oils of *Salvia* species were evaluated. Anti-oxidant activity was measured using the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and the 2,2,-diphenyl-1-picrylhydrazyl (DPPH) scavenging assays and compared to the results with the controls obtained Trolox® and vitamin C, respectively. Nearly all the solvent extracts displayed anti-oxidant activity with IC_{50} values ranging from 1.61 to 74.50 μg/ml using the $DPPH^{\cdot}$ radical, while the IC_{50} values ranged from 11.88 to 69.26 μg/ml with the $ABTS^{\cdot+}$ radical. The solvent extract of *S. schlechteri*, with an IC_{50} value of 1.61 μg/ml, was three times more active than the reference compound, vitamin C (IC_{50} value: 4.72 μg/ml). All the essential oils displayed poor activity with all the IC_{50} values being greater than 100 μg/ml using both the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals. Total phenolic content based on gallic acid equivalents (GAE) confirmed the presence of total soluble phenolics in the various extracts from 45 to 211 mg of GAE dry sample and showed strong association (r^2 = 0.90) with anti-oxidant activity (DPPH assay).

6.1 Introduction

Recent developments in biomedical science emphasise the involvement of free radicals in many diseases. There is increasing evidence to suggest that many degenerative diseases such as brain dysfunction, cancer, heart disease and immune system decline could be the result of cellular damage caused by free radicals and that anti-oxidants may play an important role in disease prevention (Aruoma, 1998). Anti-oxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. The anti-oxidant activity of natural substances is based on their ability to donate hydrogen atoms to free radicals. Many phenolic compounds exhibit a wide range of biological effects including antibacterial and anti-inflammatory activity (Cook and Samman, 1996). Studies have also shown that phenolic compounds are potent scavengers of free radicals and as such, are potentially useful in the prevention of a number of diseases (Zainol *et al*., 2003). Plants are a potential source of natural anti-oxidants.

Plants have played a significant role in the development of new drugs and in many developing countries attention has been paid to exploring natural substances as substitutes for synthetic compounds. In recent years, phytochemicals have appeared in the market and are generally regarded as safe (Wanasundara and Shahidi, 1998). The commonly used anti-oxidants, butylated hydroxyanisol and butylated hydroxytolune are synthetic chemicals and the possible toxicity of these anti-oxidants has resulted in their reduced usage (Ito *et al*., 1985). Due to health concerns, natural anti-oxidants have been extensively employed in recent years (Yen *et al*., 2003).

The reported high anti-oxidant activity and use of *Salvia* in local traditional medicine prompted more extensive investigation in order to provide a better understanding of medicinal value of indigenous species. A literature survey revealed no reference to previous work on the anti-oxidant activity of *Salvia* species found in South Africa. Most studies have focused on *S. officinalis* (garden sage) because of its well-known and widely documented anti-oxidant properties (Tepe *et al*., 2006).

The objectives of this study were to:

(i) evaluate the anti-oxidant activity of South African *Salvia* species using the

$ABTS^{\cdot+}$ and $DPPH^{\cdot}$ scavenging assays,

(ii) determine the total phenolic content present in each species, and

(iii) correlate the anti-oxidant activity to the total phenolic content.

6.2 Materials and methods

6.2.1 Reagents, chemicals and standards

Dimethyl sulfoxide (DMSO) and HPLC grade methanol were purchased from Rochelle Chemicals. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and potassium persulfate ($K_2S_2O_8$) were purchased from Fluka, while the 2,2′-azino-bis (3-ethylbenzothiazoline-6-sufonic acid) (ABTS), 3,4,5-trihydroxybenzoic acid commercially known as gallic acid and the standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®) were obtained from Sigma®. Sodium hydroxide (NaOH) and vitamin C were obtained from Saarchem. Potassium dihydrogen phosphate (KH_2PO_4), Folin-Ciocalteu phenol reagent, HPLC grade ethanol and anhydrous sodium carbonate were purchased from Merck. Double distilled Millipore® (0.22 μm) water was used throughout.

6.2.2 Evaluation of the anti-oxidant activity

The anti-oxidant activity of volatile (essential oils) and non-volatile (solvent extracts) samples were evaluated using both the ABTS and the DPPH assays.

6.2.2.1 The ABTS method

The anti-oxidant activity was determined using the method described by Villaño *et al*. (2004) with slight modifications.

(i) Preparation of the $ABTS^{\cdot+}$ radical and stock solution of test samples

The ABTS solution was prepared at a concentration of 7 mM and the potassium persulfate solution at a concentration of 140 mM with double distilled water. The radical cation $ABTS^{\cdot+}$ was chemically generated with $K_2S_2O_8$ and the ABTS solution. Hence 88 μl of the $K_2S_2O_8$ solution was added to 5 ml of ABTS solution, mixed well, covered with foil and kept at room temperature for 12-16 hours before used in the experiment. The $ABTS^{\cdot+}$ radical (stable for 2-3 days) was diluted with cold ethanol and maintained on ice

throughout the experiment. The experiment was performed only if the absorbance of the radical was 0.70 ± 0.02 at 734 nm. Stock solutions of test samples were prepared at starting concentrations of 10 mg/ml in DMSO (extracts and essential oils).

(ii) The ABTS assay

From the stock solution of solvent extracts and essential oils, seven two-fold dilutions were prepared with DMSO. To a sample volume of 50 µl of each concentration, in a cuvette, a volume of 1 ml of the $ABTS^{\cdot+}$ radical was added and kept at 30 °C in a water bath for four minutes before the absorbance was recorded at 734 nm. The final concentrations ranged from 0.05 to 238.10 µg/ml. Each sample was tested in duplicate. The principle of the reaction is that the anti-oxidant compounds react with the $ABTS^{\cdot+}$ radical. During the reaction, the solution becomes clear or decreases in colour intensity, thus decreasing the absorbance. The mechanism of reaction of the $ABTS^{\cdot+}$ radical is shown below (Figure 6.1). The percentage of decolourisation was calculated from (Eq. 6.1) using a programme written on Microsoft Excel®. The colour interference of the test sample was not taken into consideration because there was very little variation.

$$\% \text{ Decolourisation} = \frac{100 \times (1 - \text{Abs test sample})}{\text{Abs control}} \qquad \text{(Eq 6.1)}$$

Where: Abs = absorbance at 734 nm

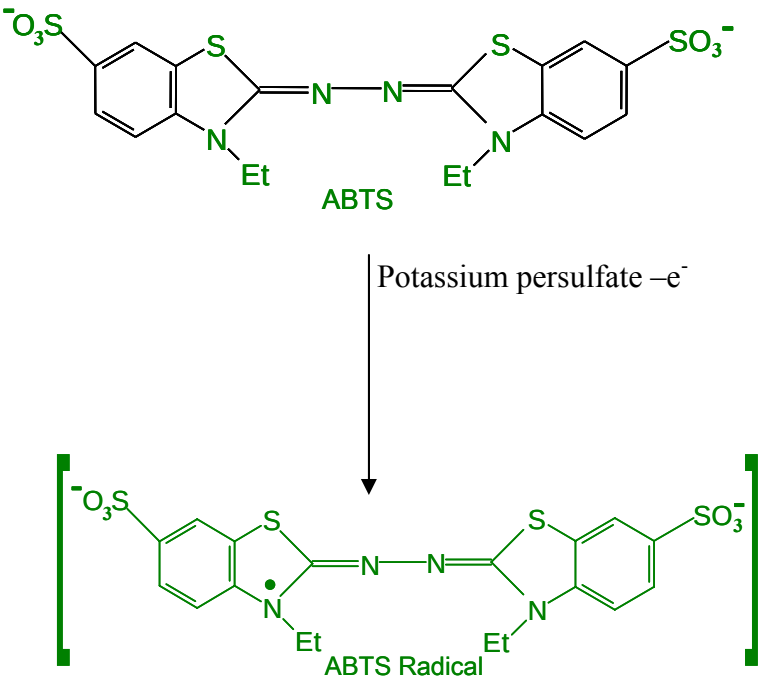
6.2.2.2 The DPPH method

(i) Preparation of the DPPH· radical and stock solution of test samples

The DPPH· radical (96 µM) was prepared in HPLC grade methanol. The radical, protected from light and stored at 4 °C (stable for approximately a week), has an absorbance of 0.38 ± 0.03 at 550 nm. The stock solution of the test sample (essential oils and solvent extracts) was prepared at a concentration of 10 mg/ml in DMSO.

(ii) The DPPH assay

The DPPH· radical is a stable radical that can readily undergo reduction by an anti-oxidant (Lu and Foo, 2001). Because of the ease and convenience of this reaction, it now has widespread use in the free radical-scavenging activity assessment (Sanchez-Moreno *et al*., 1998; Lu and Foo, 2001; Mambro *et al*., 2003). A DPPH assay (Mambro *et al*., 2003; Lourens *et al*., 2004) was also employed to investigate the anti-oxidant activity of different plant extracts and essential oils.



(peaks at 645, 734, and 815 nm)

Figure 6.1 Mechanism of action of the $ABTS^{\cdot+}$ radical (Dorman *et al*., 2000).

From the stock solution of the test sample, a 1:20 dilution was made with DMSO followed by five consecutive two-fold dilutions. For each concentration, 50 µl of the test sample was plated out in triplicate in a 96 well-plate with the appropriate control for the test sample (in order to compensate for the colour interference of the test sample). For the DPPH and methanol controls, 50 µl of DMSO was added into the wells. A volume of 200 µl of DPPH solution was added to the test samples (including the DPPH control wells with only DMSO), while 200 µl of HPLC grade methanol was added to the methanol control wells including the methanol control wells with only DMSO). The final concentrations of the test samples in the wells ranged from 3.13 to 100 µg/ml. However, due to their high activity, 12 dilutions were made for the reference compound as well as for *S. schlechteri*. The plate was then shaken at 960 rpm for 2 minutes in a microtitre plate reader (Labsystems Multiskan RC connected to a computer equipped with Genesis® version 3.03 software) and incubated in the dark, at room temperature, for 30 minutes. The principle of the method is that anti-oxidant compounds donate electron(s) to $DPPH^{\cdot}$ (Figure 6.2 and 6.3) resulting in decolourisation that is stoichiometric with respect to the electrons captured by DPPH (Hristea *et al*., 2002).

The absorbance was read after incubation using a spectrophotometer (Labsystems Multiskan RC) equipped with Genesis® version 3.03 software at a single wavelength of 550 nm and the percentage of decolourisation was determined using a simple programme written in Microsoft Excel® (Eq. 6.2).

$$\% \text{ Decolourisation} = \frac{100 \times (\text{Abs control} - (\text{Avg test Abs} + \text{Avg Abs methanol})}{\text{Abs control}} \quad \text{(Eq. 6.2)}$$

Where: Abs = absorbance

Avg = average

Abs control = Average absorbance DPPH – Average absorbance methanol

Avg test Abs = Average absorbance obtained in the wells containing DPPH and test sample

Avg Abs methanol = Average absorbance obtained in the wells containing methanol and test sample (no DPPH)

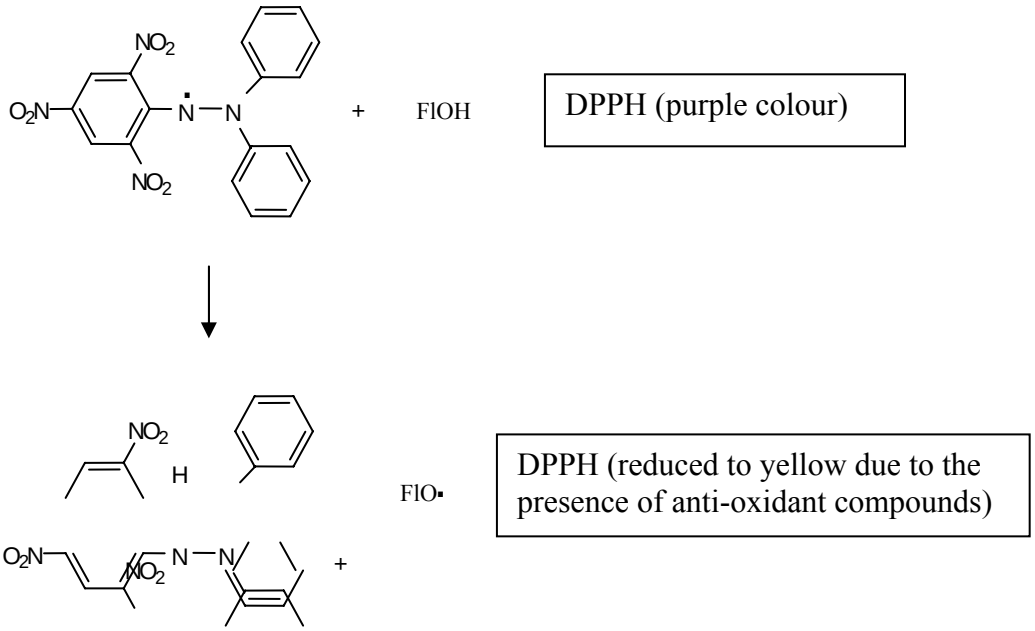


Figure 6.2 Mechanism of reaction of the DPPH$^{\cdot}$ radical (Brand-Williams *et al.,* 1995): ((FlOH: flavonoid which can donate H_2; FlO: flavonoid which has donated H_2 to the DPPH$^{\cdot}$ radical).

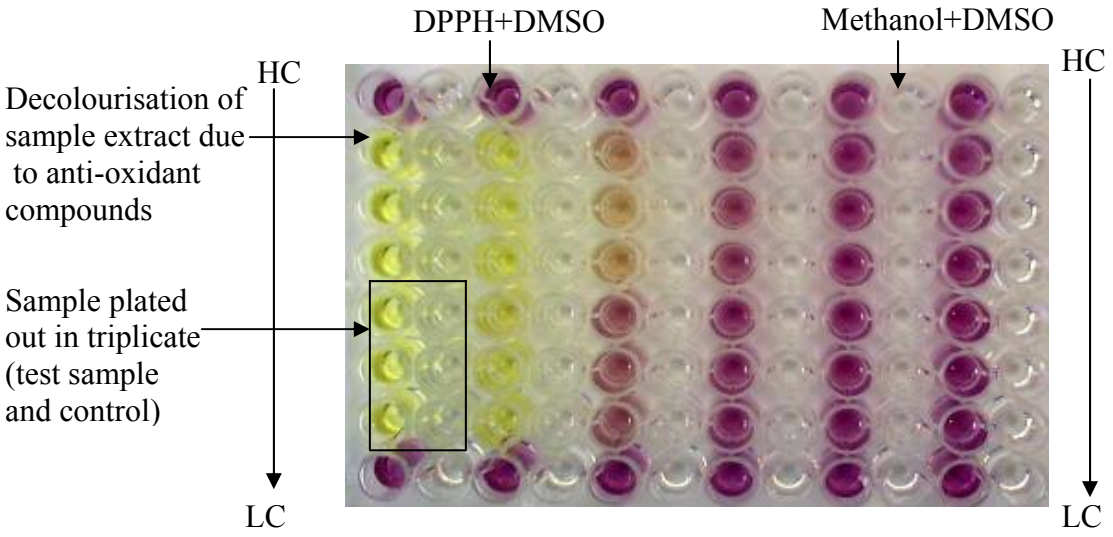


Figure 6.3 Microtitre plate showing the decolourisation of the DPPH solution in the presence of anti-oxidant compounds. HC: high concentrations of test compound with control; LC: low concentration of test compound with control, first and last row are used for DPPH and methanol controls.

6.2.2.3 Qualitative determination of the anti-oxidant activity of the solvent extracts

The anti-oxidant activity of the solvent extracts was also evaluated using a TLC method. A 3 µl sample (5 mg/ml in methanol) was spotted on two separate TLC plates (Alugram® Sil G/UV_{254}, 0.200 mm) and eluted with ethyl acetate:toluene:acetic acid (10:5:0.01). One of the TLC plates was then sprayed with 4% $DPPH^{\cdot}$ radical and another sprayed with the $ABTS^{\cdot+}$ radical (prepared with 7 mM ABTS solution and 140 mM $K_2S_2O_8$ in a ratio 3:1).

6.2.3 Determination of total phenolic content

6.2.3.1 Preparation of sodium carbonate and Folin-Ciocalteu reagent

Anhydrous sodium carbonate was prepared at a concentration of 75 g/l. The solution was stirred and heated until the sodium carbonate was completely dissolved before the solution was filtered using a Whatman® No 1 paper filter. The Folin-Ciocalteu stock solution was prepared at a concentration of 0.1% (v/v) with double distilled water.

6.2.3.2 Preparation of standard and test samples

Gallic acid was used as the standard to quantify the phenolic content in the test samples. The stock solution and subsequent dilutions (concentration ranged from 10 to 250 mg/ml)

were prepared with DMSO. The test samples (4 mg) were dissolved in 4 ml of DMSO and filtered through Whatman® No 1 filter paper.

6.2.3.3 Method for determining total phenolic content

The total phenolic content of the solvent extracts was determined by the method using Folin-Ciocalteu and gallic acid as standards (Slinkard and Singleton, 1977; Chandler and Dodds, 1983). A 2 ml volume test sample was mixed with 10 ml of Folin-Ciocalteu reagent followed by the addition of 8 ml of anhydrous sodium carbonate. After incubation at room temperature in the dark for 2 hours, the absorbance of the reaction mixture was read twice at 765 nm against a sample containing only DMSO, using a double beam spectrophotometer (Hitachi).

6.3 Data analysis

For both the ABTS and DPPH assays, the IC_{50} values (concentration at which 50% of decolourisation is obtained) were determined from the log sigmoid-dose response curves generated by the Enzfitter® version 1.05 software (Figure 6.4). Gallic acid was used as a standard to produce the calibration curve for total phenolic content (Figure 6.5).

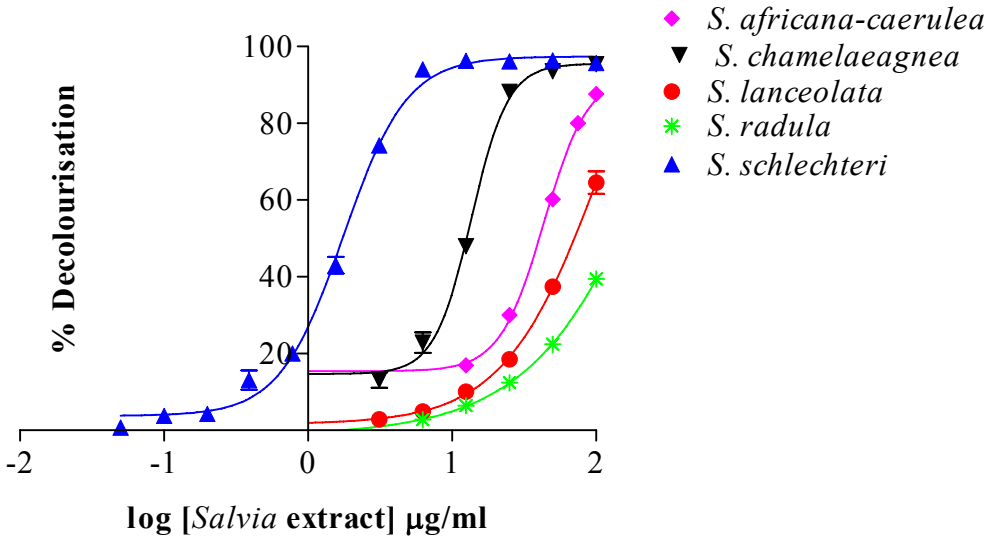


Figure 6.4 Free radical scavenging activity of five *Salvia* species against the DPPH˙.

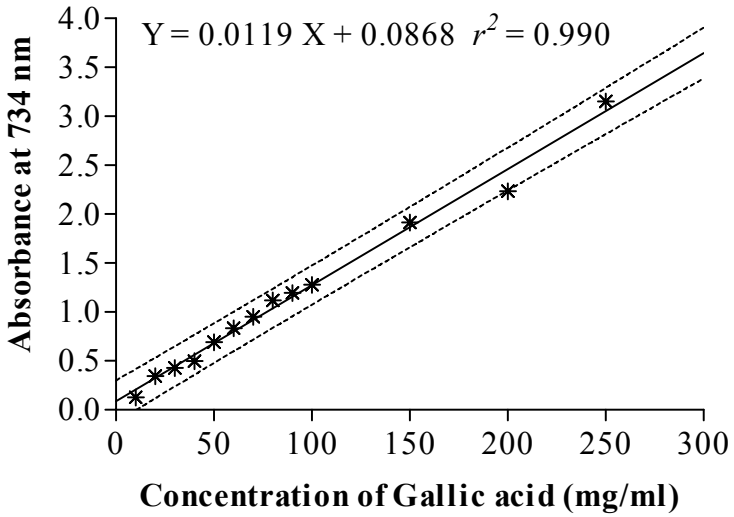


Figure 6.5 Calibration curve of gallic acid used to estimate the total phenolic content in solvent extracts, plotted with a 95% confidence interval.

To determine the total phenolic content, the mean of two readings was recorded and the quantification expressed in mg/g of gallic acid equivalent dry sample (mg/g of GAE). The correlation between the anti-oxidant activity and total phenolic content was determined using the linear regression equation. Trolox® and vitamin C were used as standard anti-oxidant compounds against the ABTS and DPPH methods, respectively. All results of the anti-oxidant activity were obtained from three independent experiments and given as mean ± standard deviation (s.d.). Experimental data were analyzed using the Student t-tests. with Statistica version 5.0 and Prism® version 3.0 software. Throughout the analysis, $P < 0.05$ was considered significant.

6.4 Results

6.4.1 Qualitative evaluation of the anti-oxidant activity of the solvent extracts

The qualitative analysis of the solvent extracts showed that similar compounds are responsible for the anti-oxidant activity when the $ABTS^{\cdot+}$ or the $DPPH^{\cdot}$ radicals were used (Figure 6.6). Anti-oxidant compounds are indicated by the white spots on the green background ($ABTS^{\cdot+}$ radical) or purple background ($DPPH^{\cdot}$ radical). The two TLC plates also showed that the anti-oxidant compounds can be polar or less polar. However, the majority of the white spots have relatively low R_f values implying that the anti-oxidant compounds were mostly polar.

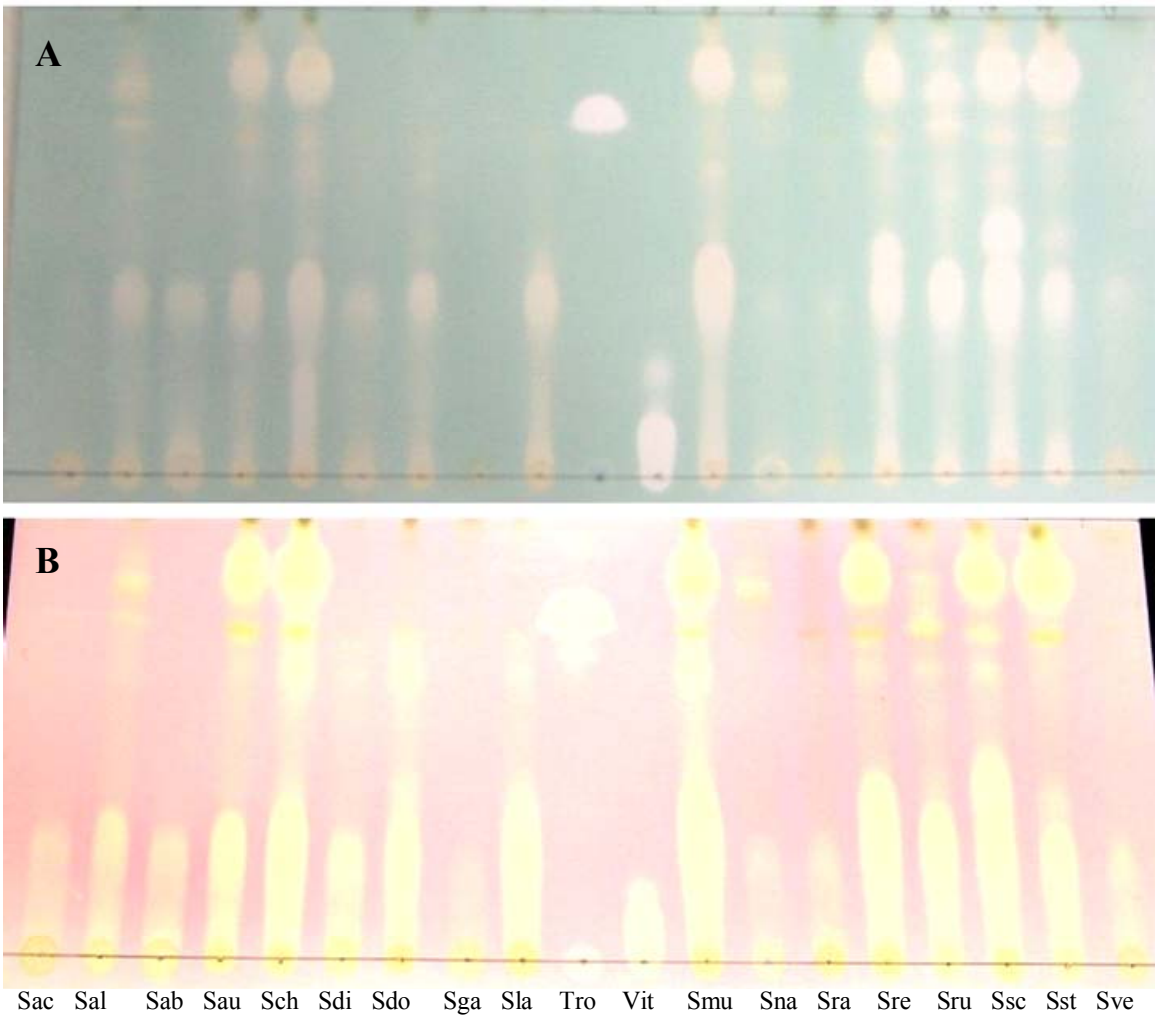


Figure 6.6 Qualitative determination of the anti-oxidant activity of the solvent extracts: (**A**): $ABTS^{\cdot+}$ radical and (**B**) $DPPH^{\cdot}$ radical: Sac: *S. africana-caerulea*, Sal: *S. africana-lutea*, Aab: *S. albicaulis*, Sau: *S. aurita*, Sch: *S. chamelaeagnea*, Sdi: *S. disermas*, Sdo: *S. dolomitica*, Sga: *S. garipensis*, Sla: *S. lanceolata*, Tro: Trolox®, Vit: Vitamin C, Smu: *S. muirii*, Sna: S. *namaensis*, Sra: *S. radula*, Sre: *S. repens*, Sru: *S. runcinata*, Ssc: *S. schlechteri*, Sst: *S. stenophylla*, Sve: *S. verbenaca*.

6.4.2 The quantitative determination of the anti-oxidant activity

Several solvent extracts displayed potent activity against both the $ABTS^{\cdot+}$ and the $DPPH^{\cdot}$ radicals (Table 6.1), while all the essential oils were poorly active and were excluded from Table 6.1. The IC_{50} values ranged from 11.88 ± 1.52 to 69.26 ± 3.22 µg/ml and from 1.61 ± 0.03 to 74.15 ± 2.85 µg/ml against the ABTS and DPPH assays, respectively.

Table 6.1 The anti-oxidant activity of solvent extracts determined by the ABTS and DPPH assays (mean ± s.d., $n = 3$) and the total phenol content.

Species/Controls	ABTS assay	DPPH assay	Total phenol content
	IC_{50} values in µg/ml		(mg/g of GAE)
S. africana-caerulea	39.72 ± 1.76	33.40 ± 3.73	115.06
S. africana-lutea	30.35 ± 2.97	47.58 ± 2.61	67.83
S. albicaulis	24.22 ± 1.28	19.85 ± 1.01	100.27
S. aurita	22.94 ± 2.57	16.59 ± 0.22	119.09
*S. chamelaeagnea**	14.61 ± 2.25	12.75 ± 1.04	211.78
S. disermas	33.05 ± 2.86	55.07 ± 4.01	69.01
S. dolomitica	49.94 ± 1.17	> 100	53.04
S. garipensis	41.66 ± 2.71	74.15 ± 2.85	45.56
S. lanceolata	25.63 ± 0.66	68.09 ± 3.69	54.22
*S. muirii**	11.88 ± 1.52	11.06 ± 0.52	186.15
S. namaensis	16.57 ± 2.56	10.63 ± 0.75	190.5
S. radula	69.26 ± 3.22	> 100	55.73
S. repens	18.24 ± 1.87	15.47 ± 1.75	178.17
*S. runcinata**	19.42 ± 2.78	19.34 ± 0.61	149.34
S. schlechteri	17.51 ± 2.05	1.61 ± 0.03	209.18
S. stenophylla	20.84 ± 0.86	14.92 ± 0.93	161.28
S. verbenaca	37.47 ± 1.77	45.83 ± 2.78	73.29
Trolox®	2.43 ± 0.07	2.51 ± 0.41	─
Vitamin C	─	4.72 ± 0.14	─

* denotes no significant difference between the ABTS and the DPPH methods

─ not determined

The solvent extract of *S. schlechteri* displayed the most favorable activity against the $DPPH^{\cdot}$ radical, which was statistically more active than the reference compound, vitamin C ($P < 0.05$), while the extract of *S. muirii* was the most active against the $ABTS^{\cdot+}$ radical. The extracts of *S. radula* and *S. dolomitica*, which were poorly active (IC_{50} values > 100 µg/ml) using the $DPPH^{\cdot}$ radical, were moderately active against the $ABTS^{\cdot+}$ radical (IC_{50} values: 69.26 ± 3.22 and 49.94 ± 1.17 µg/ml, respectively) (Figure 6.7; Table 6.1). Although the two methods measure the percentage decolourisation of two radicals, there is

a statistically significant difference in IC_{50} values generated by the ABTS and the DPPH methods (Figure 6.7) (Table 6.1). The ABTS method in general gave lower IC_{50} values compared to the DPPH method (Table 6.1). However, the Student *t*-tests did not show significant differences between the IC_{50} values of *S. chamelaeagnea*, *S. muirii* and *S. runcinata* ($P > 0.05$).

None of the 11 essential oils tested displayed promising anti-oxidant activity with either the ABTS or DPPH methods. All the IC_{50} values obtained were greater than 100 µg/ml.

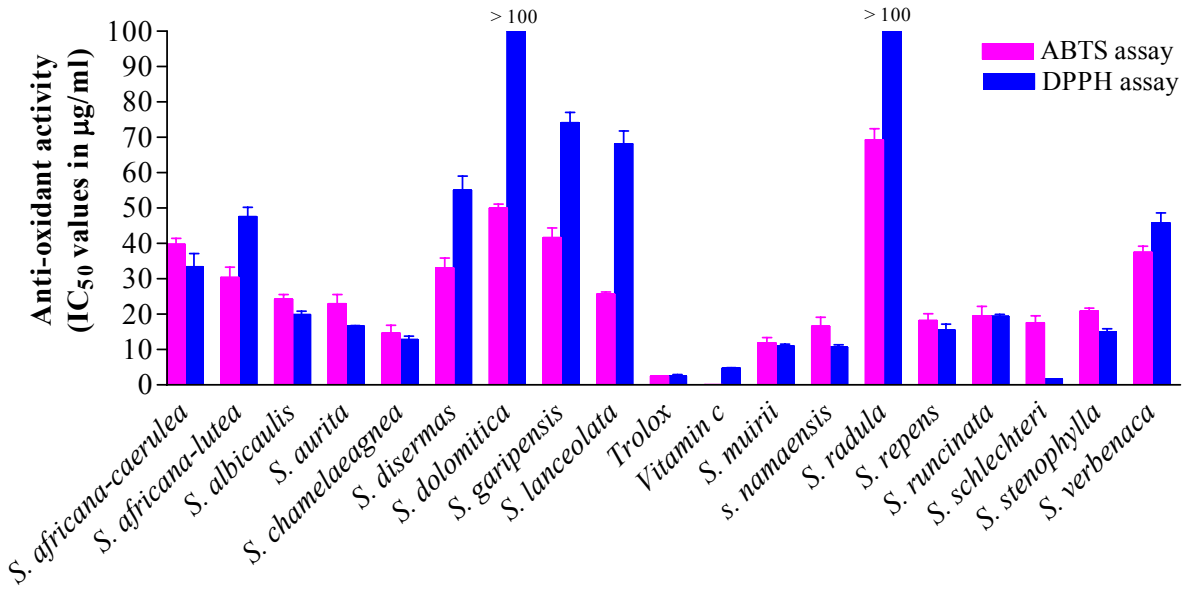


Figure 6.7 Comparison between the anti-oxidant activity of the solvent extracts of *Salvia* species using the ABTS and the DPPH assays: Sac: *S. africana-caerulea*, Sal: *S. africana-lutea*, Aab: *S. albicaulis*, Sau: *S. aurita*, Sch: *S. chamelaeagnea*, Sdi: *S. disermas*, Sdo: *S. dolomitica*, Sga: *S. garipensis*, Sla: *S. lanceolata*, Tro: Trolox®, Vit: Vitamin C, Smu: *S. muirii*, Sna: S. *namaensis*, Sra: *S. radula*, Sre: *S. repens*, Sru: *S. runcinata,* Ssc: *S. schlechteri*, Sst: *S. stenophylla*, Sve: *S. verbenaca*.

6.4.3 Total phenolic content and its relationship with the anti-oxidant activity

The anti-oxidant activity exhibited by the solvent extracts depends on the type of compounds present. A calibration curve used to estimate the total phenolic content is displayed in Figure 6.5. The phenolic values estimated in the organic extracts of the various species ranged from 45.56 to 211.78 mg/g of GAE dry sample (Table 6.1). Species such as *S. chamelaeagnea*, *S. muirii, S. repens*, *S. runcinata, S. schlechteri* and *S.*

stenophylla with high phenolic content (> 150 mg of GAE) had the most promising anti-oxidant activity (Table 6.1).

After inverse transformation of IC_{50} values obtained (to accommodate the anti-oxidant capacity from low to high values on the X-axis), there was a stronger correlation between the total phenolic content and the anti-oxidant activity for the DPPH assay compared to the ABTS assay (r^2 = 0.90 and 0.76, respectively) (Figure 6.8). This implies that the phenolic compounds are partly, if not totally, responsible for the anti-oxidant activity observed in the species studied.

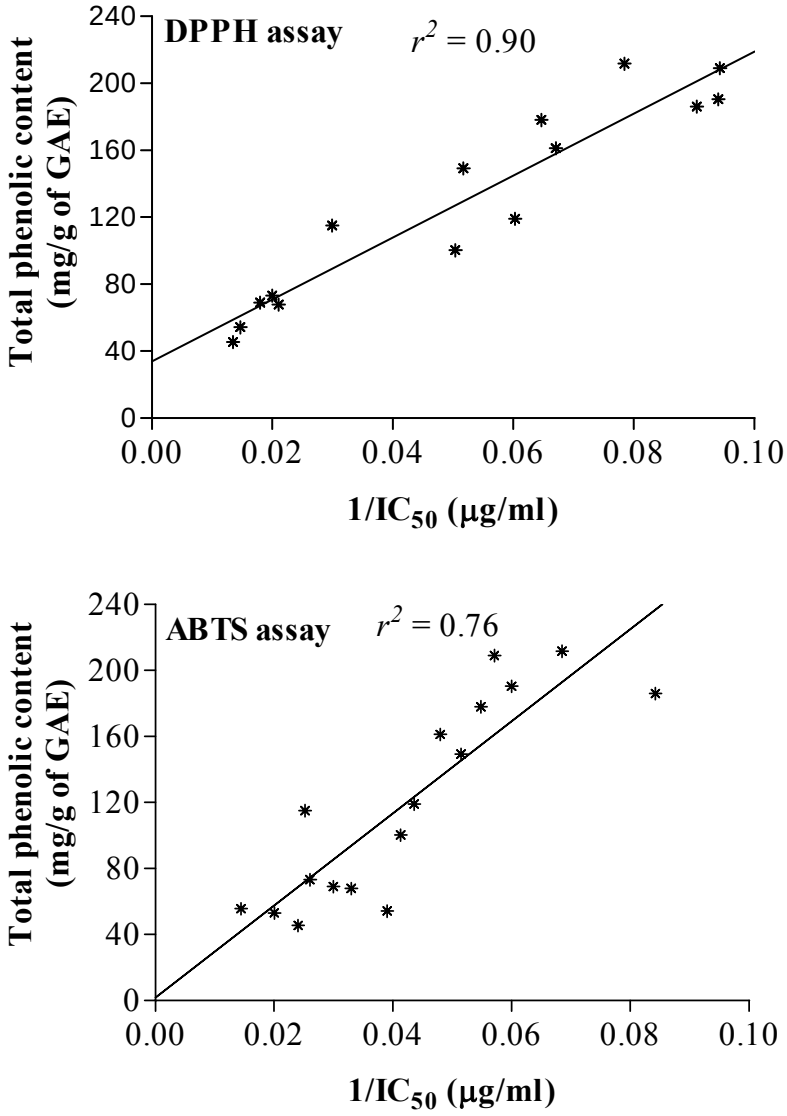


Figure 6.8 Regression analysis of the anti-oxidant activity plotted against the total phenolic content using the DPPH and ABTS methods.

6.5 Discussion

The majority of solvent extracts exhibited potent anti-oxidant activity against the $ABTS^{\cdot+}$ and the $DPPH^{\cdot}$ radicals (IC_{50} values < 75 µg/ml) with the exception of *S. dolomitica* and *S. radula* which showed weak activity in the DPPH assay (Table 6.1). The solvent extract of *S. schlechteri* was more active than vitamin C. In contrast, all the essential oils were poorly active (IC_{50} values > 100 µg/ml) in both assays. The results obtained in the present study are in agreement with previous investigations on *Salvia* species. *S. tomentosa* (IC_{50} value: 18.7 µg/ml), *S. hypageia* (IC_{50} value: 34.6 µg/ml), *S. sclarea* (IC_{50} value: 23.4 µg/ml) and *S. caepitosa* (IC_{50} value: 41.3 µg/ml) have been reported to display anti-oxidant activity against the $DPPH^{\cdot}$ radical (Tepe *et al*., 2005, Tepe *et al*., 2006). The anti-oxidant activity of *S. officinalis* has also been reported (Cuvelier *et al*., 1994). However, as far as the literature survey has ascertained, there are no published reports on the anti-oxidant activity of southern African *Salvia* species. *Salvia dolomitica* and *S. radula* demonstrated weak anti-oxidant activity against the $DPPH^{\cdot}$ radical and moderate activity against the $ABTS^{\cdot+}$ radical.

The differences obtained between the two methods indicated that the DPPH assay recorded lower anti-oxidant activity as compared to the ABTS assay. A similar incongruence has been reported previously between the two methods (Kim *et al*., 2002) and these differences may be attributed to absorbance interference at 550 nm by other compounds in the DPPH assay. In addition, the ABTS method is very sensitive, requiring a shorter reaction time (four minutes) compared to 30 minutes for the DPPH method. It can be used in both organic and aqueous solvent systems (Lee *et al*., 2003). Therefore, the ABTS assay may be preferable to the DPPH method for evaluating the total anti-oxidant activity of a given sample (Lee *et al*., 2003).

The total phenolic content (Table 6.1) found in the species was high and varied from 45.56 to 211.78 mg/g of GAE dry sample and a strong correlation using the DPPH method was obtained between the anti-oxidant activity and the total phenolic content (Figure 6.6). It is well known that *Salvia* species contain various phenolic compounds including flavonoids, rosmarinic acid, caffeic acid, carnosol and carnosic acid (Lu and Foo, 2002; Tepe *et al*., 2006). The anti-oxidant activity of sage has been attributed to the phenolic compounds (Cuvelier *et al*., 1994, Cuvelier *et al*., 1996; Hohmann *et al*., 1999). Tepe *et al*. (2005) demonstrated that *S. tomentosa*, which exhibited good anti-oxidant activity, also possessed

a high phenolic content (200 µg/mg). Several reports have conclusively shown a close relationship between total phenolic content and anti-oxidant activity (Velioglu *et al*., 1998; Deighton *et al*., 2000). In the present study it was found that *S. chamelaeagnea* and *S. schlechteri* contained a substantial amount of phenolics, that are at least partly, if not entirely, responsible for the anti-oxidant activity (Table 6.1). As can be observed from the present data, anti-oxidant activity does not necessarily correlate with high amounts of phenolics as seen by the moderate correlation between the anti-oxidant activity using the ABTS method and the total phenolic content (Figure 6.8) (Kähkönen *et al*., 1999).

The primary anti-oxidant activity of rosemary and sage is related to the presence of carnosic acid (Wenkert *et al*., 1964). It was also reported that carnosic acid and carnosol are responsible for 90% of anti-oxidant activity of rosemary (Cuvelier *et al*., 1996). Caffeic acid, rosmarinic acid, ursolic acid, oleanolic acid, carnosic acid and carnosol are common in many plants and all are strong radical scavengers. These compounds and derivatives have been shown to display anti-oxidant activity comparable to the reference compound vitamin C. Carnosol (isolated from *S. chamelaeagnea*, Chapter 9), standards of caffeic acid and rosmarinic acid also exhibited good anti-oxidant activity using the DPPH assay with the IC_{50} values 6.10 ± 0.6; 2.55 ± 0.17, 2.48 ± 0.20 µg/ml, respectively. The HPLC data (Chapter 5) revealed the presence of rosmarinic acid, caffeic acid carnosol and derivatives (carnosic acid) in the majority of the extracts. For example, *S. aurita*, *S. chamelaeagnea*, *S. muirii*, *S. repens, S. runcinata* and *S. schlechteri* with the presence of caffeic acid, carnosol, rosmarinic acid and/or carnosic acid exhibited good anti-oxidant activity, while those lacking these compounds were poorly active (e.g. *S. dolomitica*, *S. radula* and *S. verbenaca*). These compounds might therefore account for the anti-oxidant activity obtained in most investigated species.

Although the chemical composition and structures of active extract components are important factors governing the efficacy of natural anti-oxidants, the anti-oxidant activity of an extract cannot be explained based only on their phenolic content without further characterization (Heinonen *et al*., 1998). Studies have indicated that the anti-oxidant activity of phenolic acids is related to their structure. It has been reported that phenolic compounds with *ortho*- and *para*-dihydroxylation or a hydroxy and a methoxy group are more effective than simple phenolics (Frankel *et al*., 1995).

It has been reported that polar extracts exhibited stronger activity than non-polar extracts (Duan *et al*., 1998; Tepe *et al*., 2005; Tepe *et al*., 2006). For instance, if an electron donating group, especially a hydroxyl group, is located on the *o*- or *p*- positions of the phenolic compound, it makes the compound polar and therefore anti-oxidant activity is increased (Duan *et al*., 1998). The strong anti-oxidant activity of some *Salvia* species could be attributed to these groups that enable phenols to donate hydrogen atoms easily, thereby disrupting the chain-reaction of auto-oxidation. Weng and Wang (2000) showed that the anti-oxidant activity of *S. plebeia* was concentrated in the acidic, weakly acidic and phenolic fractions of the extract, while the activity of neutral fractions was negligible.

$DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radical scavenging activity of the essential oils was very low (IC_{50} values > 100 µg/ml) and this was obviously related to their chemical composition. In addition, essential oils have a low phenolic content (Cuvelier *et al*., 1994, Cuvelier *et al*., 1996). Essential oils are generally non-polar and many studies have shown that the anti-oxidant activity is associated with polar fractions (Tepe *et al*., 2005; Tepe *et al*., 2006). Other members of the Lamiaceae also showed poor activity against the $DPPH^{\cdot}$ radical. For instance, Şahin *et al*. (2006) demonstrated that *Origanum vulgare* spp. *vulgare* was poorly active against the $DPPH^{\cdot}$ radical (IC_{50} value > 8900 µg/ml). In contrast, Tepe *et al*. (2004) demonstrated that *S. multicaulis* and *S. crypthantha* essential oils were more active than the reference anti-oxidant compounds such as vitamin C and butylated hydroxy toluene (IC_{50} values of 2.4 and 3.9 µg/ml, respectively). The GC-MS data (Chapter 4) indicated that the essential oils were mostly devoid of constituents generally displaying anti-oxidant activity such as eugenol, thymol, carvacrol and γ-terpinene (Baratta *et al*., 1998; Ruberto and Baratta, 2000, van Zyl *et al*., 2006). However, these individual components of the oils were generally tested at only one concentration (1000 µg/ml). The essential oils found in the current study may also reveal some degree of activity at 1000 µg/ml, but this value is above the cut-off used to evaluate the anti-oxidant activity of samples in this study. It was found that sesquiterpene hydrocarbons exerted a low, if any, anti-oxidant effect, while monoterpene hydrocarbons showed a significant protective effect with several variants due to the functional group (Ruberto and Baratta, 2000; Dorman *et al*., 2000). The quantitative difference in their amount, as well as the antagonism in the mixture of essential oils may also explain why the activity was low. Furthermore, there are various methods for anti-oxidant activity determination of a given sample such as linoleic acid peroxidation, ferric thiocynate and thiobarbituric acid methods and perhaps the essential oils may display

greater activities using another method. The studies reported here provide initial results on the anti-oxidant properties of South African indigenous *Salvia* species. In order to investigate whether good anti-oxidant extracts can also exhibit good anti-inflammatory properties, the anti-inflammatory activity of *Salvia* species is discussed in the next Chapter.

6.6 Conclusions

- Indigenous *Salvia* species are a good source of anti-oxidants as they are rich in phenolic compounds.
- The $DPPH^{\cdot}$ radical was less sensitive than the $ABTS^{\cdot+}$ radical.
- Similar compounds are responsible for the anti-oxidant activity of the solvent extracts against the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals; however the degree of activity varies significantly for some species
- *S. schlechteri* and *S. muirii* were the most active solvent extracts against the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals, respectively.
- The activity of the solvent extracts of *S. schlechteri* was 3 times greater than that of the reference compound, vitamin C.
- Although a correlation between the anti-oxidant activity using the DPPH method and phenolic content has been confirmed, however the anti-oxidant activity is not always highly correlated to the total phenolic content.
- Essential oils have a poor free radical scavenging capacity against the $DPPH^{\cdot}$ and the $ABTS^{\cdot+}$ radicals.

Chapter 7: Anti-inflammatory activity-Inhibition of the 5-Lipoxygenase Enzyme

Abstract

5-Lipoxygenase is one of the key enzymes involved in the inflammation process. The anti-inflammatory activity of essential oils and solvent extracts of *Salvia* species was evaluated using the 5-lipoxygenase assay. Essential oils exhibited better anti-inflammatory activity when compared to the solvent extracts. Almost all the essential oils exhibited promising anti-inflammatory activity with IC_{50} values ranging between 22.81 and 77.32 µg/ml. The essential oil of *S. runcinata* exhibited the most favourable activity, while *S. africana-lutea* displayed the lowest activity. With the exception of *S. radula*, solvent extracts displayed poor ability to inhibit the enzyme with all IC_{50} values being greater than 100 µg/ml. The components of the oils such as β-caryophyllene, *trans*-nerolidol, α-bisabolol, α-pinene, α-limonene, linalool and its ester linalyl acetate, may be responsible for the anti-inflammatory activity of the investigated essential oils.

7.1 Introduction

Inflammation is the normal physiological response of the body that occurs when it is invaded by infective agents, responding to stimulation or when it is exposed to physical, chemical or traumatic changes (Baylac and Racine, 2003). Inflammatory reactions may be initiated by a variety of mediators including eicosanoids derived from the cyclo-oxygenase and 5-lipoxygenase pathways (Figure 7.1). The 5-lipoxygenase enzyme is the initiator of the conversion of the membrane phospholipid-derived fatty acid, arachidonic acid, into a number of compounds such as leukotrienes, which cause redness, pain and irritation.

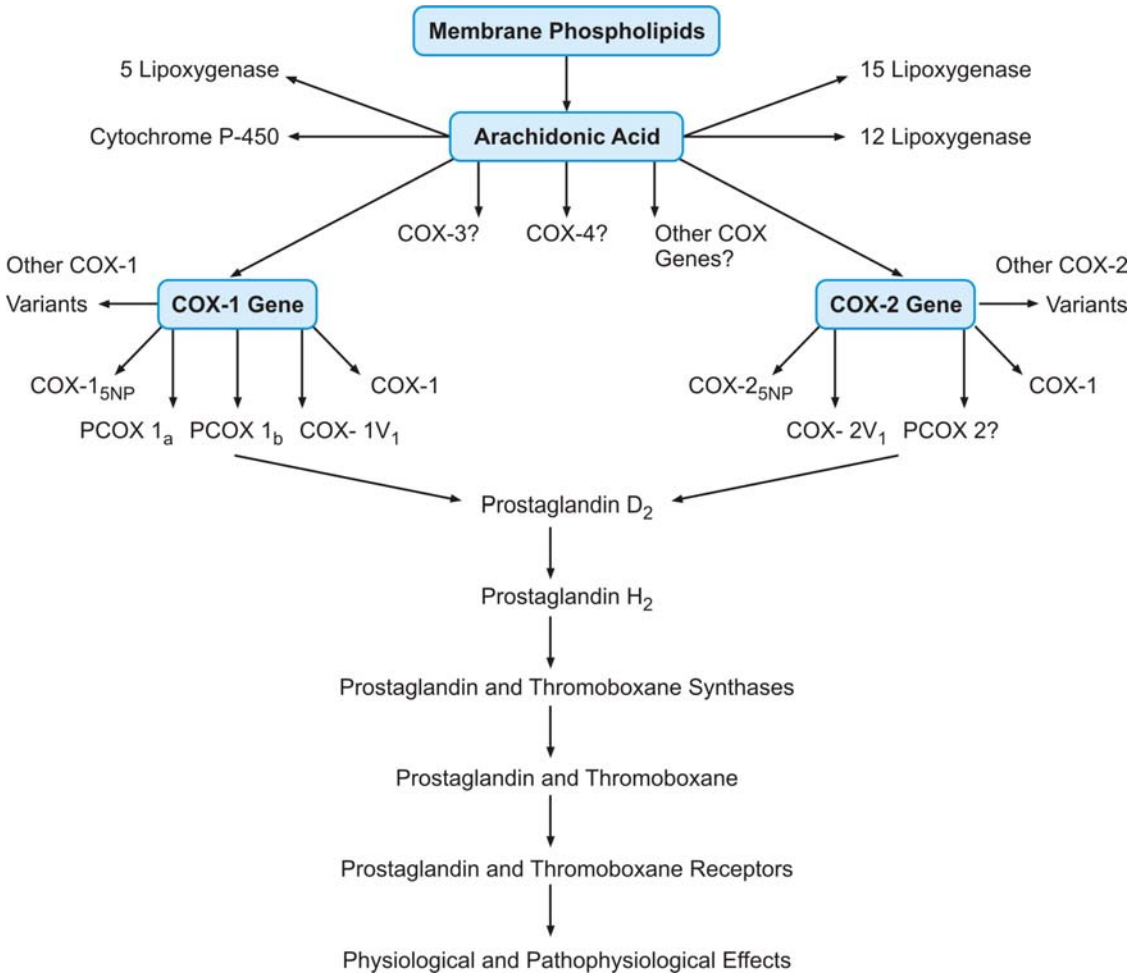


Figure 7.1 Inflammation process pathways (Davis *et al*., 2004).

Leukotrienes form a group of highly potent molecules that mediate inflammatory reactions contributing to various diseases such as asthma, cystic fibrosis, urticaria and central nervous system disorders. This important class of messengers is pro-inflammatory, resulting from the metabolism of arachidonic acid and which begins with oxidation by the

5-lipoxygenase enzyme. The biological effects of leukotrienes can be prevented by suppressing leukotriene production *via* inhibition of the lipoxygenase pathway (Figure 7.1). In recent years, a large body of evidence has accumulated, demonstrating that free radicals are important components of inflammation (Kehrer, 1993) and anti-oxidant compounds have been found to display anti-inflammatory activity (Sala *et al*., 2003). Compounds such as carnosic acid and ursolic acid which have potent anti-oxidant activity, have also been shown to possess anti-inflammatory activity (Baricevic *et al*., 2001).

As listed in Table 1.4 and 1.5, plants belonging to the genus *Salvia* have been used for various ailments and are reported to have anti-inflammatory properties. Studies have shown that the solvent extracts or isolated compounds of *Salvia* also displayed anti-inflammatory activity both *in vitro* and *in vivo* (Baricevic *et al*., 2001).

Essential oils are low molecular weight compounds which can rapidly diffuse through the skin barrier and reach the underlying tissue. They are used in many fields, such as food products, pharmaceuticals, cosmetics, perfumes and household items. Furthermore, a number of essential oils are used in aromatherapy for their therapeutic benefits (Pauli, 2001; Baylac and Racine, 2003). Previous studies carried out on *Salvia stenophylla* reported the anti-inflammatory properties of its essential oil (Baylac and Racine, 2003). Nevertheless, South African species have not yet been fully investigated. Together with the traditional uses of sage to treat body wounds (Watt and Breyer-Brandwijk, 1962), the potent anti-oxidant capacity and the reported anti-inflammatory activity of *Salvia* prompted this investigation on the anti-inflammatory activity of indigenous species.

The objective of this study was to evaluate the effectiveness of the essential oils and solvent extracts to inhibit the *in vitro* activity of the 5-lipoxygenase enzyme.

7.2 Materials and methods

7.2.1 Chemicals, reagents and standard

5-Lipoxygenase and nordihydroguaiaretic acid (NDGA) were obtained from Cayman Chemicals Company; DMSO, potassium phosphate and hydroxyl sodium from Saarchem, while Tween® 20 and linoleic acid were purchased from Fluka. Double distilled Millipore® (0.22 μm) water was used throughout this experiment.

7.2.2 Preparation of solutions, enzymes and buffers

The potassium phosphate buffer (pH 6.3) was prepared by mixing 50 ml of 0.1 M potassium dihydrogen phosphate and 9.7 ml of 0.1 M sodium hydroxyde. The solution of linoleic acid was prepared at a concentration of 0.168% in ethanol (w/v). The enzyme was aliquoted in eppendorfs (100 U ≈ 12 µl) and kept at -80 °C until used.

7.2.3 Preparation of test samples

The stock solution of the test samples (essential oils and solvent extracts) was prepared at a concentration of 10 mg/ml in the DMSO and Tween® 20 mixture and, if necessary, further dilutions were made with the same mixture. The solution of DMSO and Tween® 20 mixture was prepared by weighing 1.5 g of Tween® 20 and made up to 55 g with DMSO.

7.2.4 The 5-lipoxygenase assay

The 5-lipoxygenase assay was used as an indication of the anti-inflammatory activity. An aliquot of 50 µl of the stock solution (10 mg/ml) of each test sample was placed in a 3 ml cuvette, followed by 2.95 ml of pre-warmed potassium phosphate buffer and 48 µl of linoleic acid solution. The mixture was covered with a cap and gently shaken six times before the cuvette was placed back in the UV-VIS spectrophotometer in order to measure the absorbance reference at 234 nm. Thereafter, 12 µl of the ice-cold buffer (potassium phosphate) was mixed with 100 U of the thawed enzyme (for 10 seconds to avoid its degradation) in order to activate the enzyme. The mixture was then transferred to the cuvette and the content of the cuvette was shaken and placed back into the spectrophotometer (Specord Analytikjena-40 connected to Winaspect® software), before the absorbance was recorded at 234 nm. It is important to note that, prior to testing samples; two samples were prepared as mentioned above but only with DMSO and Tween® 20 mixture to serve as controls (no enzyme inhibition).

5-Lipoxygenase is known to catalyze oxidation of unsaturated fatty acids containing 1-4 diene (Alitonou *et al.,* 2006) and the modification of linoleic acid (diene 1-4 into diene 1-3) can be detected at 234 nm.

7.3 Data analysis

An increase in absorbance at 234 nm arising from modification of the linoleic acid (diene 1-4 into 1-3) was measured for 10 minutes at room temperature. The initial reaction determined from the slope of the linear portion (over 60 seconds) of the curve (Figure 7.2) and the percentage activity of the enzyme was calculated by comparison to the controls. The slope of the control represents 100% activity of the enzyme.

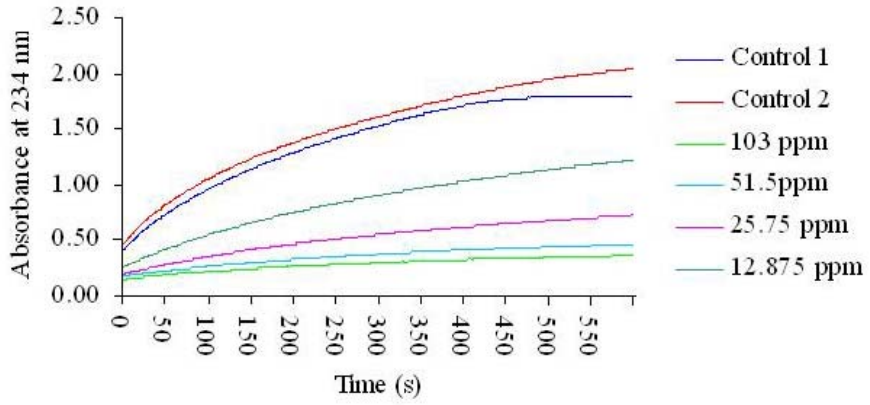


Figure 7.2 Plot of the absorbance, determined over 10 minutes, of essential oil of *Salvia runcinata* at various concentrations.

For samples exhibiting activity at 100 µg/ml, three 2-fold serial dilutions were prepared and each test sample was run once at each concentration. The concentration inhibiting 50% enzyme activity (IC_{50}) was used to evaluate the test sample and the IC_{50} value was obtained by plotting the percentage activity against the log concentration using the Enzfitter® version 1.05 software. NDGA was used as the reference anti-inflammatory compound.

7.4 Results

Results obtained for inhibition of the 5-lipoxygenase enzyme by the solvent extracts and essential oils are presented in Table 7.1. Essential oils displayed favourable activity when compared to the solvent extracts. Almost all the essential oils inhibited the 5-lipoxygenase enzyme with the IC_{50} values ranging from 22.81 ± 0.01 to 77.32 ± 6.27 µg/ml in comparison to the reference compound NDGA (IC_{50} value: 4.95 ± 0.07 µg/ml).

Table 7.1 Anti-inflammatory activity (IC_{50} ± s.e) of the essential oils and solvent extracts.

Species / Control	Essential oils	Solvent extracts
	IC_{50} values in µg/ml	
S. africana-caerulea	73.42 ± 5.27	> 100
S. africana-lutea	77.32 ± 6.27	> 100
S. albicaulis	39.22 ± 3.93	> 100
S. aurita	nd	> 100
S. chamelaeagnea	48.58 ± 0.07	> 100
S. disermas	nd	> 100
S. dolomitica	64.58 ± 6.47	> 100
S. garipensis	nd	> 100
S. lanceolata	43.38 ± 3.52	> 100
S. muirii	> 100	> 100
S. namaensis	nd	> 100
S. radula	> 100	78.78 ± 5.89
S. repens	28.04 ± 4.50*	> 100
S. runcinata	22.81 ± 0.01	> 100
S. schlechteri	nd	> 100
S. stenophylla	49.17 ± 4.87	> 100
S. verbenaca	nd	> 100
NDGA	4.95 ± 0.07	

nd = not determined, essential oil not available

* Essential oil was obtained from Lady Grey which is different from where extract was collected

NDGA: Nordihydroguaiaretic acid

s.e.: standard error of the estimate

The essential oil of *S. runcinata* exhibited the most potent activity (IC_{50} value: 22.81 ± 0.01 µg/ml), while *S. africana-lutea* displayed the lowest activity (IC_{50} value: 77.32 ± 6.27 µg/ml). The essential oils of *S. muirii* and *S. radula* displayed poor activity at a starting concentration of 100 µg/ml.

Nearly all the solvent extracts exhibited poor anti-inflammatory activity except *S. radula* which displayed moderate activity (IC_{50} value: 78.78 ± 5.89 µg/ml). The IC_{50} values of the solvent extracts were greater than 100 µg/ml against the 5-lipoxygenase enzyme. It was

interesting to note that in contrast to poor activity of the essential oil of *S. radula*, the solvent extract was fractionally more potent, whilst *S. muirii* was found to possess no anti-inflammatory properties in either essential oil or solvent extract (Table 7.1).

7.5 Discussion

Baylac and Racine (2003) found that the essential oil of *S. stenophylla* has the potential to inhibit the *in vitro* 5-lipoxygenase enzyme ($10 < IC_{50}$ value < 30 in μg/ml). In this study, *S. stenophylla* also displayed anti-inflammatory activity (IC_{50} value: 49 μg/ml). The difference found between the two values reported, although the same method was used, may be attributed to variation in chemical composition of the two essential oils. Many factors such as harvesting time, geographical location, climate and stage of development of the plant have been reported to affect the chemical composition of the essential oil (Putievsky *et al*., 1986).

The biological activity of essential oils or plant extracts is mainly associated with their chemical composition. The essential oil data given in Chapter 4 can be used to explain the results obtained in this study. Essential oils are complex mixtures comprising of a multitude of compounds and each constituent contributes to the beneficial or adverse effect of the oils (Lahlou, 2004). It is therefore difficult to determine exactly which compound(s) is/are responsible for the biological activity when crude essential oils are used.

Aromatherapy reports on the anti-inflammatory activity of terpenes and sesquiterpenes, and data suggests that the presence of these compounds may account for the activity of the essential oils as determined by the 5-lipoxygenase *in vitro* enzyme. Sesquiterpenoids and their derivatives are credited with various biological action including anti-asthmatic, antibacterial, antifungal, anti-inflammatory and antineoplastic activities (Farnworth and Bingel, 1977). Structure-activity relationships to define broad categories of activities for the different classes of molecules found naturally in essential oils have been investigated. Terpenic hydrocarbons (α-pinene, $31 < IC_{50}$ value < 50 μg/ml), sesquiterpenic hydrocarbons (β-caryophyllene, $10 < IC_{50}$ value < 30 μg/ml), sesquiterpenic alcohols (*trans*-nerolidol, $10 < IC_{50}$ value < 30 μg/ml) and α-bisabolol, ($10 < IC_{50}$ value < 30 μg/ml) exhibited a potential ability to inhibit the 5-lipoxygenase enzyme (Baylac and Racine 2003). Peana *et al*. (2002**)** demonstrated that linalool and its ester linalyl acetate (Table

4.1) exhibited anti-inflammatory activity. These two compounds were found in *S. dolomitica* (16.6 and 19.6%, respectively) and may have partly contributed to the anti-inflammatory activity. It was reported that monoterpenes such as 1,8-cineole, α-pinene and limonene and sesquiterpenes such as β-caryophyllene also inhibit the *in vitro* 5-lipoxygenase assay (Juergens *et al*., 1998; Baylac and Racine, 2003). These compounds were present in the majority of species in varying concentrations (1,8-cineole: trace amounts to 40.5 %; α-pinene : trace amounts to 22.3 % and limonene: 0.5 to 11.6 %)(Table 4.1). All these components identified in the essential oils may have contributed to the anti-inflammatory activity of the essential oils. Furthermore, α-bisabolol, which is widely reported to have a skin soothing action, strongly inhibited the 5-lipoxygenase enzyme *in vitro* (Baylac and Racine, 2003). It is therefore expected that essential oils containing high levels of this sesquiterpenic alcohol like that of *S. runcinata* (65%) and *S. stenophylla* (26%), can be expected to inhibit 5-lipoxygenase enzyme as well. Moretti *et al*. (1997) evaluated the anti-inflammatory activity (induced by carrageenin in rats) of essential oils of *S. sclarea* and found that the oil exhibited a significant anti-inflammatory effect. The effect of the oil was correlated to the presence of linalool, α-terpineol and linalyl acetate. They also found that these constituents produce less anti-inflammatory action when administered separately than the oil *in toto* and are less active than the oxygenated fractions obtained by flash chromatography of the oil. This clearly indicated that the action of the essential oil might be determined by the synergistic interactions of its constituents.

Effective anti-oxidant treatment has also been found to be effective against inflammation (Sala *et al*., 2003). For instance, phenolic acids such as carnosic and ursolic acids, which are good anti-oxidants, were reported to be good anti-inflammatory compounds (Liu, 1995; Baricevic *et al*., 2001). However, ursolic acid exhibited poor ability to inhibit the key enzyme of leukotriene biosynthesis, namely 5-lipoxygenase (Safayhi *et al*., 1997). Only the solvent extract of *S. radula* exhibited some degree of activity, while other species were inactive at the starting concentration of 100 µg/ml (Table 7.1). In addition, the anti-inflammatory activity of the solvent extracts or isolated compounds of *Salvia* species is widely reported. For example, Baricevic *et al*., (2001) investigated the topical anti-inflammatory activity of *S. officinalis* leaves and found that the solvent extracts inhibited ear oedema in mice in a dose-dependent manner, while aethiopinone, isolated from *S. aethiopis* roots, exhibited anti-inflammatory activity (Hernández-Pèrez *et al*., 1995).

Solvent extracts of *Salvia* species are rich in flavonoids which possess potent anti-oxidant activity. Although many pentacyclic triterpenes (ursolic acid) also bind to the 5-lipoxygenase enzyme (Safayhi *et al*., 1995), the presence of an 11-keto-group and a hydrophilic function of the pentacyclic ring system are crucial for potent inhibition of 5-lipoxygenase and ursolic acid turned out to be non-inhibitory (Sailer *et al*., 1996). This compound was found in some of the solvent extracts. This suggests that the components of the extract responsible for the anti-oxidant activity are not necessarily those responsible for the anti-inflammatory activity. It is important to note that the 'quasi absence' of 5-lipoxygenase inhibitory activity in this study does not imply that the solvent extracts do not possess any anti-inflammatory activity. The activity may be observed at higher concentrations. Furthermore, the biological response of any substance may be influenced by various factors, including variation in assay methods employed (Houghton and Raman, 1998). As shown in Figure 7.1, inflammation is a very complex cascade of events involving not only the 5-lipoxygenase pathway, but also the 12- and 15-lipoxygenases, as well as cyclo-oxygenase-1, cyclo-oxygenase-2, cytochrome P_{450} and epoxygenase pathways. Further investigation involving other inflammatory routes is required in order to determine whether the solvent extracts do exert anti-inflammatory activity in other steps of the complex anti-inflammatory cascade.

7.6 Conclusions

- The essential oils exhibited the ability to inhibit the *in vitro* 5-lipoxygenase enzyme.
- The solvent extracts were poorly active and only *S. radula* inhibited the *in vitro* 5-lipoxygenase enzyme below a concentration of 100 µg/ml.
- The uses of *Salvia* species by inhalation support the traditional uses of these plants and imply that essential oils may be responsible for the activity. Furthermore, the topical use of *Salvia* species may be validated.
- None of the solvent extracts or essential oils exhibited anti-inflammatory activity comparable to nordihydroguaiaretic acid.
- The possibility that the solvent extracts may inhibit other enzymes or receptors involved in the inflammatory pathway should not be disregarded and this warrants further investigation.

Chapter 8: The Antimalarial Activity of the Essential Oils and Solvent Extracts and Isolated Compounds from *S. radula*

Abstract

The resistance of *Plasmodium falciparum* to currently used drugs has become a serious problem and efforts are being directed at obtaining new drugs with different structural features. One option favoured is the search for new plant-derived antimalarial drugs. Eleven essential oils and 17 solvent extracts of indigenous *Salvia* species used in traditional medicine against various ailments, including malaria, were subjected to pharmacological testing in order to evaluate their potential to inhibit the growth of *Plasmodium falciparum* FCR-3 strain. The investigation was conducted using the [^{3}H]-hypoxanthine radiometric method. The outcome showed that both the essential oils and the solvent extracts displayed antimalarial activity. The IC_{50} values of the essential oils ranged from 1.20 to 13.50 µg/ml and were low compared to the solvent extracts (IC_{50} values ranging from 3.91 to 26.01 µg/ml). The essential oil of *S. runcinata* was the most active killing 50% of parasites at a concentration of 1.20 µg/ml. Among the plants screened, the solvent extract of *S. radula* displayed the most favorable activity, while *S. lanceolata* was the least active. The extract of *S. radula* was selected to determine the compound(s) which may be responsible for the observed antimalarial activity since it displayed the best activity. Two compounds were isolated and characterised as betulafolientriol oxide and salvigenin. The two compounds displayed comparable or lower antimalarial activity (IC_{50} values: 4.95 and 24.60 µg/ml, respectively) than the crude solvent extract. The *in vitro* antimalarial activity supports the continued use of *Salvia* species in folk medicine.

8.1 Introduction

Not considering the future implications of the AIDS pandemic, malaria is currently one of the most important human diseases in developing countries and is still unconquered. It is the world's leading cause of fatalities among the infectious diseases (Wanyoike *et al*., 2004). Most of the lethal cases are caused by *Plasmodium falciparum*, the most virulent of the four *Plasmodium* species that infect humans and which is distributed in tropical Africa, Asia and Latin America (Karou *et al*., 2003). Despite the extensive control efforts, the incidence of the disease is not decreasing, especially in developing countries where malaria remains a major public health problem (Karou *et al*., 2003). Some 3.2 billion people live in areas at risk of malaria transmission (WHO, 2005a). An estimated 350-500 million clinical malaria episodes occur annually. *Plasmodium falciparum* induced malaria causes more than 1 million deaths each year (WHO, 2005a).

The spread of parasites resistant to agents such as chloroquine and quinine, and insecticide-resistant mosquitoes, has led to major difficulties in the treatment and control of this disease and has hence triggered the search for new drugs. One possible approach to the identification of new antimalarial drug candidates is to search for compounds or plants that reportedly cure malaria. For many centuries, plants have formed the basis of sophisticated traditional medicine systems and more recently, natural products have yielded lead compounds, especially against infectious diseases (Schwikkard and van Heerden, 2002). In South Africa and in most Third World countries, the use of medicinal plants is widespread and there is real hope of finding an alternative to synthetic drugs.

Plants are known to be the main source of drug therapy in traditional medicine (Prozesky *et al*., 2001) and the use of traditional medicinal plant preparations is transmitted verbally from one generation to the next, a process than runs the risk of losing essential information or misinformation. The recognition and validation of traditional medical practices and the search for plant-derived drugs could lead to a new strategy in malaria control. It is therefore necessary to obtain more scientific information concerning the efficacy and safety of the remedies in use, as many people already use and depend on herbal medicines for the treatment of malaria (Prozesky *et al*., 2001). Many studies have been carried out on the antimalarial activity of various plant species with some encouraging results (Prozesky *et al*., 2001; van Zyl and Viljoen, 2002). Furthermore, two of the most potent antimalarial

drugs have originated from plants: quinine from *Cinchona* trees and artemisinin from *Artemisia annua* (Gessler *et al*., 1994; Foley and Tiller, 1997; Olliaro *et al*., 2001). Nevertheless, only 20% of plants which are reported to have bioactivity have been subjected to bioassay screening (Houghton, 2001). Malaria can occur despite taking antimalarial drugs and symptoms of infection usually occur within 9 to 14 days after exposure. The general symptoms of malaria include headache, nausea, fever, cough, sweating, vomiting and flu-like symptoms. These symptoms may differ depending on the type of *Plasmodium* that is responsible for the infection (WHO, 1998). The life cycle of a malaria parasite is presented in Figure 8.1.

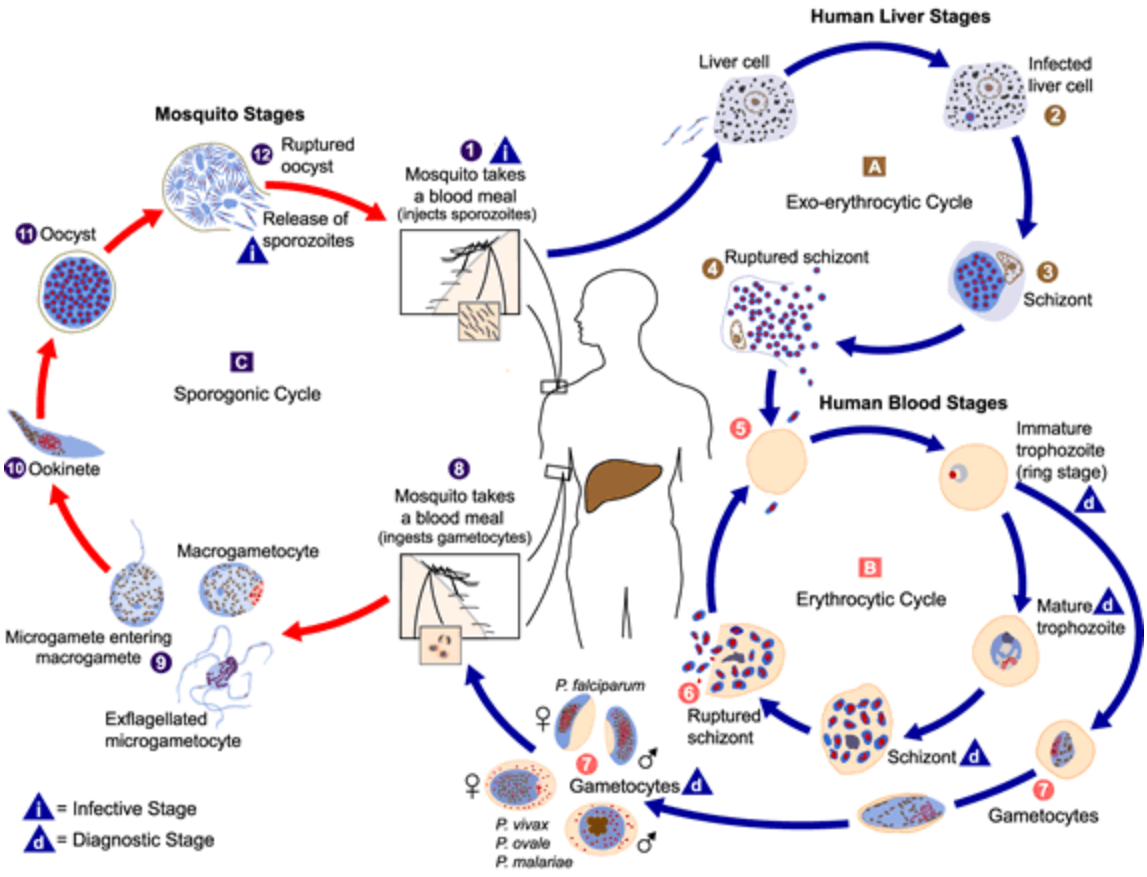


Figure 8.1 Life cycle of the malaria parasite (National Center of Infectious Disease, 2006).

The genus *Salvia* has been used in traditional medicine for the treatment of a variety of diseases, including malaria (Watt and Breyer-Brandwijk, 1962). However, no exhaustive studies on the *in vitro* antimalarial activity of these plants have been carried out on indigenous species thus far. In a continuation to verify the efficacy of traditional medicines against malaria, South African *Salvia* species were investigated.

The objectives of this study were to:

(i) study the essential oils and solvent extracts of the *Salvia* species for their potential to inhibit the *in vitro* growth of the malaria parasite, *P. falciparum*,

(ii) isolate, identify, characterize and determine the antimalarial activity of isolated compound(s) from the most promising species, and

(iii) generate reliable scientific data to validate the traditional use of these plants in the treatment of malaria.

8.2 Materials and methods

8.2.1 Chemicals, reagents and standards

Roswell Park Memorial Institute media (RPMI 1640) was purchased from Gibco™; quinine sulphate and the reagent anisaldehyde were obtained from Fluka. Gentamicin sulphate, hypoxantine (6-hydroxypurine), $NaHCO_3$ and the standard chloroquine diphospate were purchased from Sigma®, while KH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$ and glucose were obtained from Merck. [N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)] (HEPES) was obtained from Highveld Biological, while DMSO and D-sorbitol were obtained from Saarchem. Sterile water was used throughout the experiments. The [^{3}H]-hypoxanthine isotope was purchased from Amersham. All other solvents and/or reagents were of the best available grade. Silica gel was obtained from Macherey-Nagel.

8.2.2 Preparation of different solutions

8.2.2.1 Incomplete hypoxanthine negative medium and the incomplete hypoxanthine culture medium

The incomplete hypoxanthine negative medium was prepared by dissolving RPMI-1640 (10.4 g), HEPES buffer (5.9 g) and glucose (4 g) in one liter of distilled Millipore® water which was filtered through a Sterivex-GS 0.22 μm filter unit to sterilize the solution.

The incomplete hypoxanthine culture medium was prepared in the same way as the hypoxanthine negative medium except that hypoxanthine (44 mg) and gentamicin (100 μl; 50 mg/ml) were included. Both solutions were stored at 4 °C until required.

8.2.2.2 Sodium bicarbonate and phosphate buffer saline

The 5% (w/v) sodium bicarbonate was prepared by dissolving 50 g $NaHCO_3$ into one liter of distilled Millipore® water and filtered.

The phosphate buffer saline (PBS) (pH 7.4) was prepared with NaCl (8 g), KCl (0.3 g), $Na_2HPO_4 \cdot 2H_2O$ (0.73 g) and KH_2PO_4 (0.2 g) in one liter of distilled Millipore® water which was then autoclaved for 20 min at 120 °C and at 1.5 kgf/cm^2 pressure. These solutions were stored at 4 °C until required.

8.2.2.3 Complete culture medium

The complete culture medium (CCM) was prepared by supplementing the incomplete hypoxanthine negative medium (85.8 ml) with 10% (v/v) human plasma (10 ml) and 4.2 ml of 5% $NaHCO_3$. The CCM was prepared when required and discarded when it had turned dark pink which indicated that it was too alkaline for use.

8.2.2.4 Preparation of human plasma and blood

The plasma pooled from healthy donors (South African Blood Transfusion Services) was inactivated at 56 °C in a water bath for two hours, centrifuged at 3000 rpm for 20 minutes, aliquoted into sterile 50 ml centrifuge tubes and stored at -20 °C until required.

The whole blood collected from healthy donors (South African Blood Transfusion Service) was preserved in citrate phosphate dextrose adenosine-1 (CPDA-1) to prevent coagulation and, when required, was washed thrice with PBS at 2000 rpm for five minutes each. The buffy coat was removed with each wash and the remaining erythrocytes stored in hypoxanthine negative medium at 4 °C until required.

8.2.2.5 D-sorbitol and Giesmsa buffer

The D-sorbitol was made at a concentration of 5% (w/v) with distilled Millipore® water, filtered and stored at 4 °C. The Giesmsa buffer was prepared by dissolving 3.5 g of KH_2PO_4 and 14.5 g of $Na_2HPO_4 \cdot 12H_2O$ in a final volume of one liter of distilled Millipore® water and autoclaved for 20 min at 120 °C and at 1.5 kgf/cm^2 pressure.

8.2.3 *Plasmodium falciparum* and *in vitro* culture maintenance

The chloroquine-resistant Gambian FCR-3 strain was cultured continuously according to the procedure described by Trager and Jensen (1976) taking into account the modifications as described by van Zyl and Viljoen (2002). The parasite culture was suspended in complete culture medium and maintained daily. The culture consisted of at least a 2-6% parasitaemia in a 5% haematocrit of parasitised and uninfected erythrocytes in CCM (Section 8.2.2.3) which was gassed with 2.06% O_2, 5.00% CO_2, 92.94% N_2 and incubated at 37 ºC. Fresh uninfected human erythrocytes (less than 3 weeks old) were added when the parasites were predominantly in the trophozoite/schizoite stages. In order to synchronise the parasites for experimental purposes, 5% (w/v) D-sorbitol was added to a predominantly ring-staged (Figure 8.2) the culture every second day (10-15 minutes) and then washed with PBS three times at 2000 rpm five minutes each. Daily visualisation of parasites was done by preparing a thin smear and the observation was made under oil immersion objective with a light microscope (Nikon). The percentage parasitaemia was then calculated using at least 10 fields counted over the length of the smear (Figure 8.2).

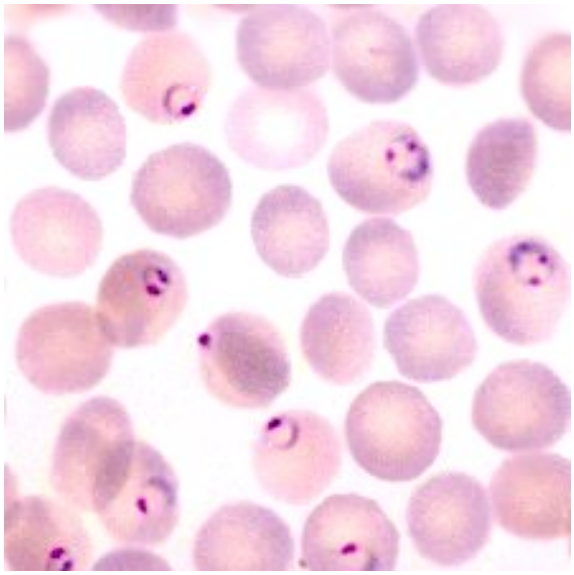


Figure 8.2 A thick blood smear showing the ring form of *P. falciparum* in erythrocytes.

8.2.4 Preparation of test samples

Stock solutions of the solvent extracts (10 mg/ml) were prepared with DMSO, aliquoted and stored at -20 °C until required. On the day of experimentation, seven dilutions of each test sample were prepared from a stock solution made with an incomplete hypoxanthine negative medium. The stock solution (10 mg/ml) and dilutions of the essential oils were

prepared on the day of the experiment with DMSO. A final concentration of 1% DMSO in the well had no effect on parasite growth.

8.2.5 Assay of antimalarial activity

The antimalarial activity was determined using the tritiated hypoxanthine radiometric assay over a single growth cycle (48 hours) (Desjardins *et al*., 1979; van Zyl and Viljoen, 2002). Before running the experiment, a thin blood film stained with diluted Giesmsa for 10-20 minutes was microscopically examined (Figure 8.2) and the percentage parasitaemia calculated (Eq. 8.1). At least 10 fields were counted over the length of the smear to obtain an average percentage parasitaemia. The percentage parasitaemia normally ranged from 2 to 6%. To prepare a suspension to be used for the *in vitro* experiment, the parasite suspension, mainly in the ring stage, was adjusted to 0.5% parasitaemia and 1% haematocrit using an incomplete hypoxanthine negative medium supplemented with 10% human plasma and 5% NaHCO3.

$$\% \text{ Parasitaemia} = \frac{\text{Number of infected cells} \times 100}{\text{Total number of infected + uninfected cells}} \qquad \text{(Eq. 8.1)}$$

For each dilution, 25 µl of solvent extract and isolated compounds, or 2 µl of essential oil, was plated out in triplicate in a 96-well culture plate (Nunclon^{TM}). In the control wells, 25 µl of incomplete hypoxanthine negative medium was plated out. The hypoxanthine negative medium (23 µl) was added to the wells containing essential oils to ensure a total volume of 25 µl in each well. Subsequently, the complete hypoxanthine negative medium containing 1% red blood cells (200 µl) was plated out in four wells and used as a control. While 200 µl of the parasitised red blood cells were added to the remaining 92 wells (each plate consists of four test samples and each test sample had seven dilutions). The plate was placed in a humidified candle jar, relatively free of oxygen and incubated for 24 hours at 37 ºC. The essential oils were always assayed in separate microtitre plates and incubated in different candle jars due to their volatile properties.

The [^{3}H]-hypoxanthine isotope (25 µl) was then added to each well and the plate was incubated at 37 ºC for an additional 24 hours. The final concentration of the solvent extracts in the wells ranged from 1 to 100 µg/ml, while the essential oil concentrations ranged from 0.05 to 50 µg/ml. Further dilutions were prepared where necessary. The

[^{3}H]-hypoxanthine was prepared by mixing 10 µl of [^{3}H]-hypoxanthine (5 mCi, 28.0 Ci/mmol) with 2.7 ml of incomplete hypoxanthine negative medium.

At the end of the second incubation period, parasite DNA was harvested onto Wallac® printed glass fiber filter mats with a 96-well semi-automated Titertek® cell harvester. The glass-fiber filter mats were left to dry overnight at room temperature and transferred to a sample bag containing 10 ml of Wallac Betaplate® liquid scintillation fluid and heat-sealed. The *in vitro* uptake of [^{3}H]-hypoxanthine by malaria parasites was used as an indicator of parasite growth. Hypoxanthine is required by parasites for DNA synthesis and is only supplied by the human host, as it is not produced *de novo*. The amount of radioactivity in counts per min (CPM) associated with the [^{3}H]-hypoxanthine incorporated into the parasite DNA trapped in the mat was determined with a β-scintillation counter (Wallac®). At least three independent experiments were carried out for each sample, with chloroquine diphosphate and quinine sulphate used as reference antimalarial drugs.

8.2.6 Isolation and structural characterization procedures from *S. radula*

S. radula was selected to isolate the compounds which may be responsible for the antimalarial activity because it showed the best activity compared to the other tested species. Dried aerial parts (723.5 g) collected on the road to Derby (North West) were powdered and exhaustively extracted with methanol:chloroform (1:1, v/v). The supernatant was then concentrated at 70 °C with a rotary evaporator under vacuum.

8.2.6.1 Isolation by column chromatography

The residue obtained (22.4 g) was mixed with a small amount of silica 60 (0.063-0.2 mm/70-230 mesh ASTM), dried at 60 °C in the oven, reduced to fine powder, added to the top of the column (4 cm x 60 cm) and eluted with hexane:dichloromethane (9:1), dichloromethane:methanol (6:1) and finally with 100% methanol, respectively. Five fractions (A-E) were collected and each fraction was tested for antimalarial activity.

Fraction C (12.5 g) displayed the best activity and was selected for further analysis. It was re-chromatographed in a smaller column (2.5 cm x 45 cm) with a solvent system of toluene:ethyl acetate (10:3). This solvent system was chosen as it displayed good separation on a TLC plate. A total number of 222 fractions in 3 ml test tubes were

collected and spotted onto a TLC plate. The separated spots on TLC were identified under UV lights (254 nm and 366 nm) and also sprayed with 0.5% anisaldehyde sulfuric acid reagent in glacial acetic acid:methanol:sulfuric acid (10:85:5). Fractions with similar R_f values were combined and concentrated to afford 7 major fractions. Fractions 3 and 5 were selected based on the results of their biological activity. Fraction 3 was eluted with toluene:ethyl acetate (10:3) and 189 sub-fractions (in 3 ml test tubes) were collected and combined into two major fractions resulting in the isolation of compound **1** (29.4 mg) with hexane:ethyl acetate:toluene (1:1:1) as eluent. Compound **2** (24.1 mg) was obtained after repeating column chromatography three times with toluene:ethyl acetate (10:2) from fraction 5. The isolation procedure of the two compounds is summarized in Figure 8.3.

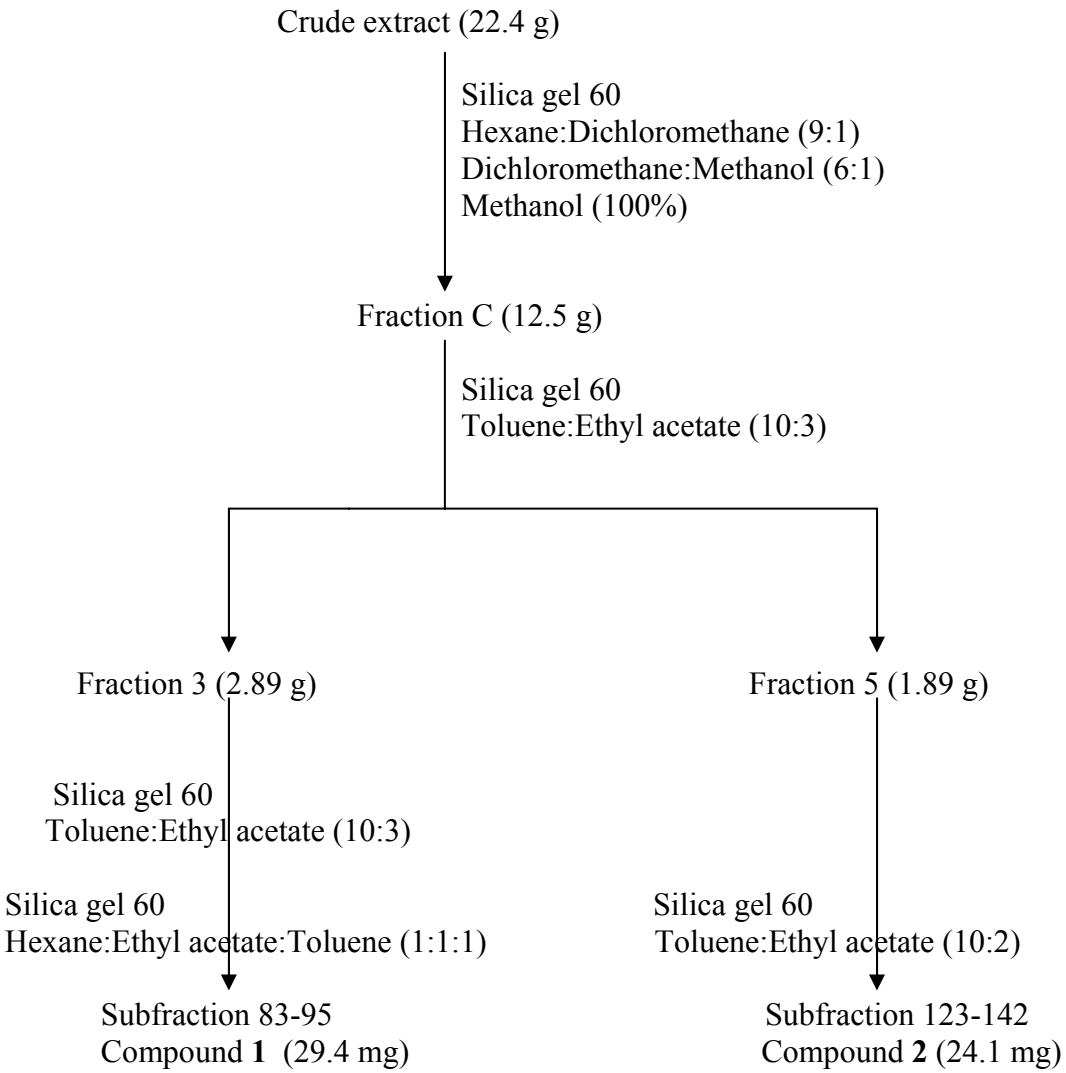


Figure 8.3 Isolation procedures of the two compounds from *S. radula*. (NB: Fractions 1, 2, 4 and 6 had low activity and were not considered for further analysis).

The isolated compounds were structurally characterized using nuclear magnetic resonance (NMR) and mass spectrometry (MS) and data were compared to those found in the literature.

8.2.6.2 Structural characterization

(i) Nuclear magnetic resonance spectroscopy

^{1}H, ^{13}C, HMBC and COSY nuclear magnetic resonance (NMR) data of compounds **1** and **2** were recorded on a Varian Inova 2000 300 MH_Z spectrometer. All spectra were recorded at 25 °C in deuterated chloroform and the chemical shifts were recorded in ppm referenced to tetramethylsilane (TMS).

(ii) Mass spectrometry

Electron ionization MS of the isolated compounds were performed by direct inlet at 70 eV on the GC-MS QP 2010 gas chromatography-mass spectrometry.

8.3 Data analysis

The percentage inhibition of radio-activity incorporation or growth (relative to the positive control values of *P. falciparum* without test sample) was calculated based upon the mean radio-active contents (CPM) of the experimental wells using a simple programme written in Microsoft Excel® (Eq. 8.2).

$$\% \text{ parasite growth} = \frac{\text{CPM (test compound sample)} - \text{CPM (mean of RBC control)} \times 100}{\text{CPM (mean of PRBC control)} - \text{CPM (mean of RBC control)}} \quad \text{(Eq. 8.2)}$$

Where: CPM: Counts per minute

RBC: Red blood cells

PRBC: Parasitised red blood cells

The log sigmoid dose-response curve was then constructed using the Enzfitter® version 1.05 and Prism® version 3.0 software and the IC_{50} values (the concentration required to kill 50% parasite) calculated as a measure of the antimalarial activity of the solvent extracts, isolated compounds and essential oils (Figure 8.4). The Student t-tests and one-way analysis of variance (ANOVA) were used to compare data. A linear regression was used in order to determine any possible relationship between the activity of the solvent extract and the essential oil of the same plant. For all statistical analyses, $P < 0.05$ was considered

significant. The IC_{50} values are given as the mean ± s.d. of three independent experiments. All the statistical analyses were performed using the Statistica® version 5.0 software.

8.4 Results

8.4.1 The antimalarial activity of the essential oils and solvent extracts

The results of the *in vitro* tests of the essential oils and solvent extracts against chloroquine-resistant *P. falciparum* (FCR-3) are presented in Table 8.1. The essential oils and extracts of the evaluated species were found to exhibit antimalarial activity at IC_{50} values of less than 30 µg/ml. The activity of essential oils (IC_{50} values) ranged from 1.20 ± 0.08 to 13.50 ± 2.20 µg/ml. Among the plants tested, the essential oil from *S. runcinata* was significantly the most active (IC_{50} value: 1.20 ± 0.08 µg/ml) ($P < 0.05$).

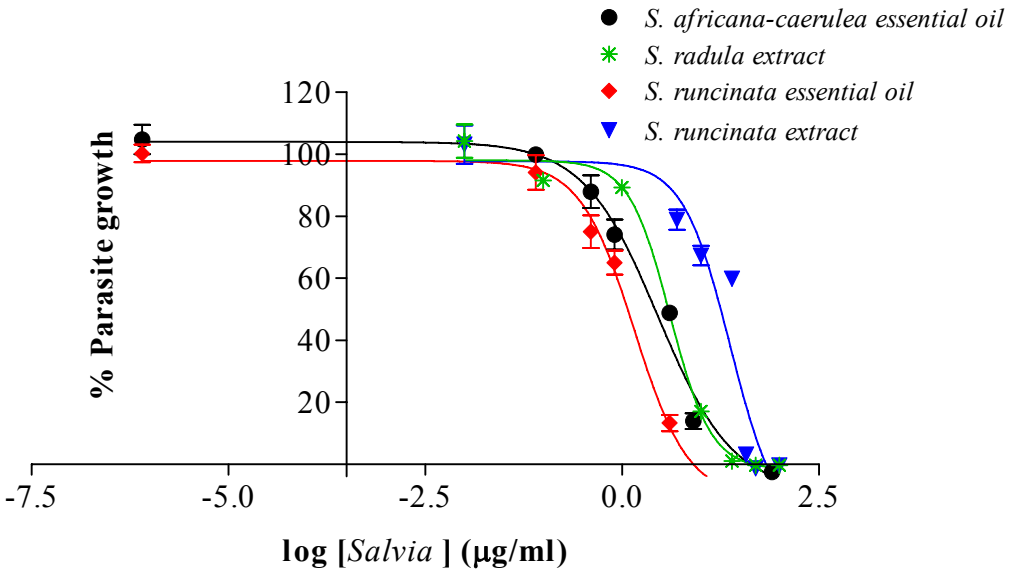


Figure 8.4 Antimalarial properties of the solvent extract of *Salvia radula*, *S. runcinata* and the essential oils of *S. africana-caerulea* and *S. runcinata*.

An ANOVA showed that the essential oils of five species namely *S. africana-caerulea*, *S. africana-lutea*, *S. albicaulis*, *S. dolomitica*, *S. muirii* and *S. stenophylla* exhibited similar antimalarial activity ($P > 0.05$). The antimalarial activity of the solvent extracts (IC_{50} values) ranged from 3.91 ± 0.52 to 26.01 ± 2.95 µg/ml. The extract of *S. radula* was the most active (IC_{50} value: 3.91 ± 0.52 µg/ml) ($P < 0.05$).

Table 8.1 *In vitro* antimalarial activity of the solvent extracts and essential oils of indigenous *Salvia* species and isolated compounds (mean ± s.d., $n = 3$).

Species/Isolated/ reference compounds	Essential oils	Solvent extracts
	IC_{50} values in µg/ml	
*S. africana-caerulea**	4.76 ± 0.48	22.68 ± 2.80
*S. africana-lutea**	5.45 ± 0.35	15.86 ± 5.04
*S. albicaulis**	6.41 ± 1.97	15.83 ± 1.94
S. aurita	nd	8.92 ± 2.63
*S. chamelaeagnea***	8.63 ± 0.96	8.71 ± 1.96
S. disermas	nd	24.17 ± 4.10
S. dolomitica *	4.81 ± 0.74	7.62 ± 1.44
S. garipensis	nd	13.95 ± 3.76
*S. lanceolata**	7.83 ± 0.98	26.01 ± 2.95
*S. muirii**	5.93 ± 1.03	11.87 ± 2.13
S. namaensis	nd	25.38 ± 2.11
S. radula	13.50 ± 2.20	3.91 ± 0.52
*S. repens**	1.65 ± 0.26***	8.25 ± 2.09
*S. runcinata**	1.20 ± 0.08	16.61 ± 3.33
S. schlechteri	nd	17.51 ± 2.05
*S. stenophylla**	4.13 ± 0.63	6.50 ± 1.37
S. verbenaca	nd	23.97 ± 1.10
Compound **1**	4.95 ± 2.00[a]	
Compound **2**	24.60 ± 1.38[a]	
Chloroquine diphosphate	0.06 ± .01	
Quinine sulphate	0.14 ± 0.03	

*Denotes activity of the essential oil is greater than activity of the solvent extract

* *Denotes no significant difference between the activity of the solvent extract and essential oil (P >0.05)

*** Essential oil obtained from Lady Grey (not from the same location as the solvent extract)

[a]: mean ± s.e. ($n = 1$) due to the small quantity of the isolated compound

nd: Not determined, essential not available

One-way analysis of variance showed that there was no difference in the antimalarial activity of the solvent extracts of *S. aurita*, *S. chamelaeagnea*, *S. dolomitica*, *S. repens* and *S. stenophylla* (P > 0.05). The activity of *S. africana-lutea*, *S. albicaulis*, *S. garipensis*, *S.*

muirii, *S. runcinata* and *S. schlechteri* were also not different (P > 0.05). Finally, *S. africana-caerulea*, *S. disermas*, *S. lanceolata*, *S. namaensis* and *S. verbenaca* also exhibited the same activity statistically (P > 0.05).

Comparison of the activity of the volatile (essential oils) and non-volatile fractions showed significant differences with the volatile fractions generally exhibiting higher activity (P < 0.05) (Figure 8.5). It was interesting to note that *S. radula,* with the lowest activity among the essential oils (IC_{50} value: 13.50 ± 2.20), displayed the best activity of the solvent extracts (Figure 8.1) (Table 8.1). In addition, there was no statistical difference between the activity of the solvent extract and essential oil of *S. chamelaeagnea* (P > 0.05). The rest of the species showed significant differences between the activity of the essential oil and solvent extract, with the activity of the essential oil being greater than that of the solvent extracts (P < 0.05).

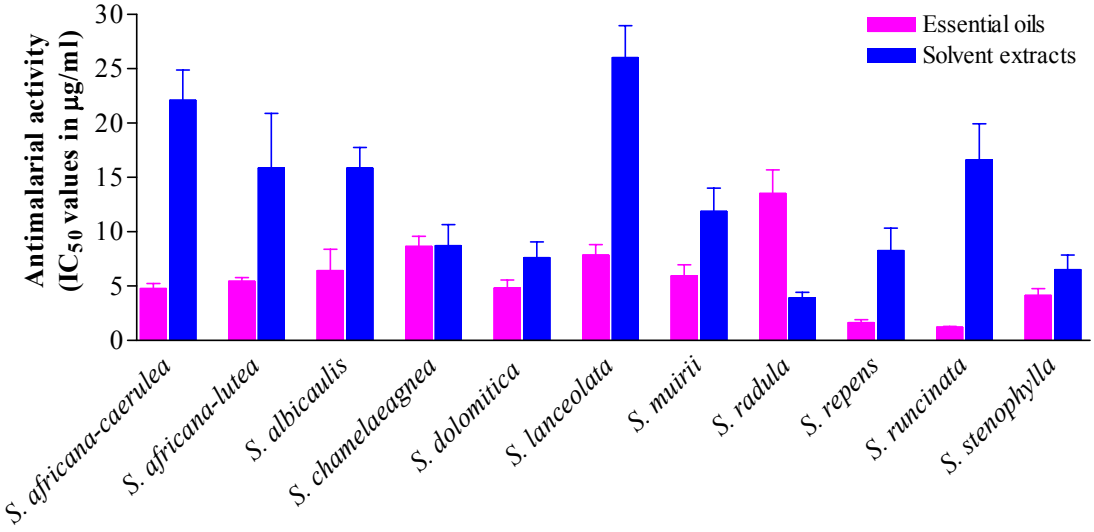


Figure 8.5 Comparison between the antimalarial activity of the essential oils and the solvent extracts of eleven *Salvia* species (mean ± s.d., *n* = 3).

The activity of the essential oils and solvent extracts was low compared to the reference antimalarial drugs such as chloroquine and quinine (P < 0.05) (Table 8.1). The essential oil of *S. runcinata* (IC_{50} value: 1.20 µg/ml) was 20 and 9 times less active than the reference antimalarial drugs chloroquine and quinine, respectively. Similarly, the activity of *S. radula* (IC_{50} value: 3.91 µg/ml) was 65 and 28 times less active than chloroquine and quinine, respectively.

The very poor correlation (r^2 = 0.04) (Figure 8.6) between the antimalarial activity of the essential oils and solvent extracts implies that the compounds responsible for the activity in the essential oils were most probably different to those found in the solvent extracts.

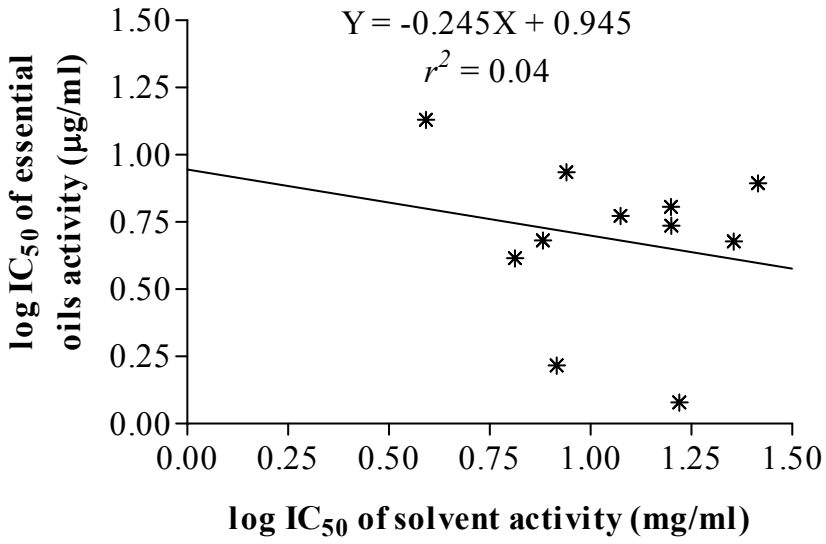


Figure 8.6 Relationship between the antimalarial activity of the solvent extract and essential oil of the same plant.

8.4.2 Identification and characterization of the isolated compounds

The ^{1}H-NMR and the ^{13}C-NMR of the isolated compounds are displayed in Table 8.2 and Table 8.3 and were compared to the data found in the literature (Voirin, 1983; Harraz *et al*., 1995; Rouf *et al*., 2001).

The ^{1}H and ^{13}C spectra of the two isolated compounds are in Appendix D. Compound **1** was isolated as a white anhydrous powder. From the mass spectrum, compound **1** has a M^+ at m/z 476 which corresponds to a molecular formula of $C_{30}H_{52}O_4$. This compound**,** a triterpene, was identified as betulafolientriol oxide.

Table 8.2 NMR data (in $CDCl_3$) of compound **1** isolated from *Salvia radula*.

Position	δ_C	δ_H
1	33.6	
2	25.4	
3	76.2	3.39, br, s
4	37.6	
5	49.6	
6	18.2	
7	34.6	
8	40.0	
9	50.3	
10	37.3	
11	31.2	
12	71.0	3.51, td, *J* = 10.6, 4.7 Hz
13	49.4	
14	52.1	
15	32.6	
16	28.6	
17	47.9	
18	15.4	0.98, s
19	16.1	0.86, s
20	86.5	
21	27.6	1.26, s
22	31.2	
23	25.0	
24	85.4	3.84, dd, *J* = 9.0, 6.7 Hz
25	70.1	
26	26.1	1.09, s
27	27.9	1.27, s
28	28.3	0.93, s
29	22.0	0.83, s
30	18.2	0.91, s
OH		5.58, s; 3.89, s

$CDCl_3$: chloroform, s: singlet, d: doublet, dd: double of doublets, td: triple of doublets, t: triplet, br: broadened, δ_H: proton shift, δc: carbon shift: *J*: coupling constant, Hz: hertz.

Compound **2** was isolated as a yellow anhydrous powder, and was determined to be a flavonoid known as hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one (salvigenin) with a molecular formula of $C_{18}H_{16}O_6$.

The UV and chromatographic data of betulafolientriol oxide and salvigenin are shown in Figure 5.1 and Table 5.1, while the structures of the two compounds are presented in Figure 8.7.

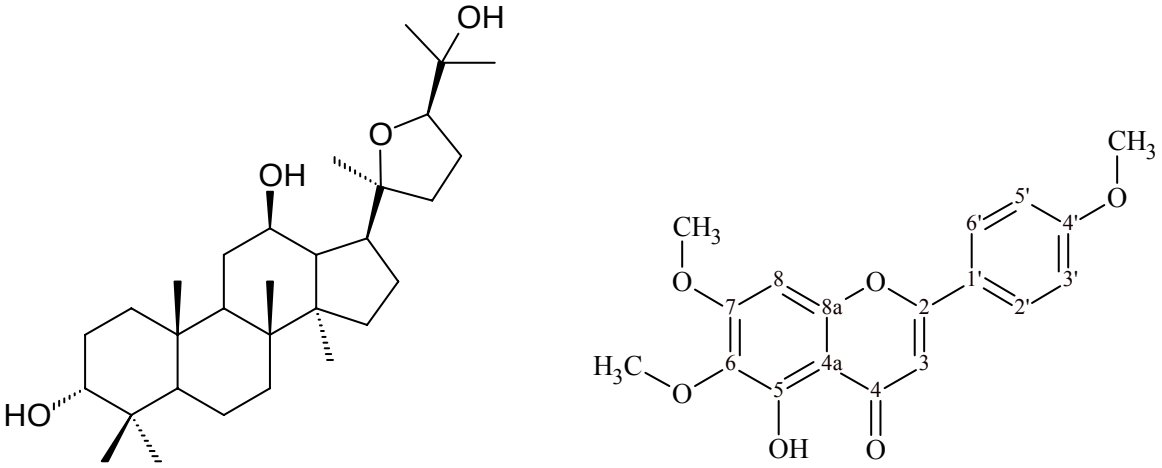
Table 8.3 NMR data (in $CDCl_3$) of compound **2** isolated from *Salvia radula*.

Position	δ_C	δ_H	Correlations observed in HMBC
2	162.6		
3	104.1	6.59, s	C-1',4
4	182.7		
4a	106.1		
5/8a	153.2		
6	132.6		
7	158.7		
8	90.5	6.55, s, H-8	C-4,6
8a/5	153.1		
1'	123.6		
2',6'	128.0	7.85, d, *J* = 9.0 Hz	C-2',4',6'
3',5'	114.5	7.02, d, *J* = 9.0 Hz	C-1',2,3',5'
4'	164.0		
6-OCH_3	60.8	3.93, s	C-6
7-OCH_3	56.3	3.97, s	C-7
4'-OCH_3	55.5	3.89, s	C-4'
5-OH		12.77, s	C-4a, 5, 6

$CDCl_3$: chloroform, s: singlet, d: doublet, δ_H: proton shift, δc: carbon shift: *J*: coupling constant, Hz: hertz, HMBC: heteronuclear multiple bond correlation.

The two isolated compounds were tested for antimalarial activity. Betulafolientriol oxide exhibited the best activity compared to the flavanoid (salvigenin) with 5 µg/ml killing 50% of parasites (Table 8.1). Although statistical comparison was not possible (because the

isolated compounds were tested only once due to the small amount obtained through the isolation procedure), salvigenin was less active, while betulafolientriol oxide exhibited comparable activity to the crude extract of solvent *S. radula*. the two compounds were less active than some essential oils and the reference antimalarial drugs (Table 8.1).



(1) Betulafolientriol oxide ($C_{30}H_{52}O_4$)

(2) 5-Hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one ($C_{18}H_{16}O_6$) (Salvigenin)

Figure 8.7 Structures of the isolated compounds from *Salvia radula*.

8.5 Discussion

Malaria is a worldwide problem and the increasing spread of *P. falciparum* strains that are resistant to standard treatment has initiated numerous studies aimed at identifying new antimalarials. Traditional medicine seems to be a very important source of active molecules (Bodeker, 2000). Indeed, at this moment, compounds from plant origin, such as quinine or its alkaloids and, more recently, artemisinin and its derivatives, are the most efficient drugs available to combat malaria (Price *et al*., 1998). The essential oils and organic extracts from *Salvia* species in this study exhibited antimalarial properties against the *P. falciparum* FCR-3 strain with all IC_{50} values lower than 30 µg/ml. These results confirm that species belonging to the genus *Salvia* contain metabolites with considerable antimalarial potential against *P. falciparum*.

Essential oils were significantly more active than the solvent extracts ($P < 0.05$). Essential oil constituents have low molecular weights and can easily diffuse through the cell

membrane. This may partly explain why they are more active than the solvent extracts. The direct application of an essential oil in the treatment of malaria has not yet been considered. However, the presence of potential antimalarial compounds in the essential oils, as well as solvent extracts, might open possibilities for further investigation in the field of antimalarial compounds (Valenti *et al*., 1995).

The biological activity of the solvent extracts and essential oils depends on the type of compounds present in each fraction. For instance, the antimalarial activity of essential oils may be attributed to their high sesquiterpene content. Sesquiterpenoids and their derivatives are credited with numerous biological properties, including antimalarial activity. Recently, nerolidol (an acyclic oxygenated sesquiterpene) from the leaves of *Virola surinamensis* (Rol.) Warb. was identified as an active component of an essential oil that displayed antimalarial activity (Lopes *et al.,* 1999). Furthermore, parasites treated with nerolidol showed decreased ability to synthesize co-enzyme Q in all intra-erythrocytic stages (Boyom *et al*., 2003). van Zyl *et al*. (2006) evaluated the antimalarial activity of twenty individual commercial essential oil constituents (alcohols, aldehydes, esters, ketones, phenols and terpene hydrocarbons). Nerolidol, linalyl acetate, pulegone and α-pinene were amongst the most active constituents (IC_{50} values < 0.3 μg/ml). α-Pinene is present in the majority of essential oils (traces-22.3%), while linalyl acetate was only detected in *S. dolomitica* (19.6%) (Table 4.1). The antimalarial activity of many essential oils from other plant families is well reported in literature. The essential oils of *Tetradenia riparia* (Lamiaceae) obtained from the leaves and stems exhibited moderate antimalarial activity against chloroquine-sensitive D10 and the chloroquine-resistant strain FAC-8 (IC_{50} values: 25.5 and 50.3 μg/ml, respectively). Major components of the oils were α-terpineol, fenchone, β-fenchyl alcohol and perillyl alcohol and these oil components were absent in the current study and may explain the difference in the antimalarial activity (Campbell *et al*., 1997). Valenti *et al*. (1995) investigated the antimalarial activity of *Lippia multiflora* (Verbenaceae) and found that the essential oils also displayed activity. The major components of the oil, nerolidol (45.2%) and linalool (20.2%), were thought to be responsible for the activity. Nyiligira (2004) investigated the antimalarial activity of *Vitex* species against the chloroquine-resistant FCR-3 and found that the oils were also active (IC_{50} values < 9.3 μg/ml). Although the essential oils studied comprise a multitude of compounds, it can be speculated that the presence of the compounds listed above and identified in indigenous *Salvia* essential oils may have contributed to the activity observed.

It is important to note that not all essential oils exhibit antimalarial properties. For instance, Milhau *et al*. (1997) studied the activity of the essential oil of *S. officinali*s and found that the activity was very poor (IC_{50} value > 1000 µg/ml). This poor activity may, however, be attributed to the fact that a different parasite strain was used and the chemical composition of the oil was also different.

Clarkson *et al*. (2004) demonstrated that *S. repens* displayed antimalarial activity against *P. falciparum* (IC_{50} value: 10.8 µg/ml). In this study, the IC_{50} value of *S. repens* was 8.25 ± 2.09 µg/ml which is similar to that obtained by Clarkson *et al*. (2004).

Phenolic compounds are credited with various biological activities including anticancer, antibacterial, anti-oxidant activities. Oliveira *et al*. (2004) demonstrated that the antimalarial activity of *Bidens pilosa* (Asteraceae) was mainly correlated to its flavonoid content. In Chapter 5, the HPLC analysis revealed the presence of flavonoids in the solvent extracts of the investigated species. This class of compound may have also contributed to the antimalarial activity of the solvent extracts.

Alkaloids also exhibited antimalarial activity against *P. falciparum* (Sanon *et al*., 2003). Alkaloids isolated from *Sida acuta* (Malvaceae) were also tested for antimalarial activity and results pointed out that they were 18 times more active than the solvent extract (Karou *et al*., 2003). Although no research was done in this study to confirm the presence of alkaloids in species investigated, alkaloids have previously been identified in some indigenous *Salvia* species, such as *S. chamelaeagnea*, *S. namaensis* and *S. runcinata* (Raffauf, 1996).

Salvia species are traditionally used to treat fever, cough and diarrhoea, all symptoms of malaria infection. This study showed the *in vitro* efficacy of *Salvia* species against *P. falciparum*, the causative organism. The isolated compounds from *S. radula,* and the presence of betulafolientriol oxide (IC_{50} value: 5 µg/ml) in all the investigated species (HPLC analysis, Chapter 5), may have contributed to the overall activity of the solvent extracts. Betulafolientriol oxide may act alone or in combination with other compounds present to determine the overall activity of the solvent extract.

It has also been demonstrated that methoxylated aromatic compounds, such as compound **2,** display antimalarial activity (Kraft *et al*., 2002). In this study, salvigenin (compound **2**) was not as active the crude extract or reference antimalarial drugs (Table 8.1). Phytochemical studies of plants used to treat the symptoms of malaria in traditional medicine of different countries revealed the presence of methoxylated aromatic compounds (Reddy *et al*., 2003). Furthermore, the methoxylated flavonoids have been found to enhance the *in vitro* activity of artemisinin (Liu *et al*., 1989; Bilia *et al*., 2002).

The activity of the isolated compounds was low compared to that of the crude extract (Table 8.1). Williamson (2001) demonstrated that the compound known as ginkgolides, isolated from *Ginko biloba,* was less effective than the crude extract. The activity of the crude extract may be the result of a multitude of compounds present in the solvent extracts acting in a synergistic manner rather than the effect of only one compound.

8.6 Conclusions

- *Salvia* species exhibit antimalarial properties against *P. falciparum* FCR-3.
- Generally, the activity of the volatile fraction was statistically greater than that of the non-volatile fractions.
- Betulafolientriol oxide and hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one isolated from *S. radula* also displayed antimalarial activity, but the activity of the two compounds was low compared to the antimalarial activity of the crude extract.
- The essential oils and solvent extracts were less active than chloroquine diphosphate and quinine sulfate.
- This preliminary study provides a scientific basis for the traditional use of *Salvia* species in treating malaria, although more thorough *in vivo* evaluation is required.

Chapter 9: Antibacterial and Antimycobacterial Activities of *Salvia* Species and Isolated Compounds from *S. chamelaeagnea*

Abstract

Salvia species are commonly used in traditional medicine to treat various microbial infections. Essential oils and solvent extracts of South African *Salvia* species were investigated for their antibacterial and antimycobacterial activities using the micro-dilution and BACTEC^{TM} 460 radiometric methods, respectively. The micro-organisms included two Gram-positive (*Staphylococcus aureus* and *Bacillus cereus*), two Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacterial strains, as well as the organism responsible for tuberculosis, *Mycobacterium tuberculosis*. With the exception of *S. radula*, all the solvent extracts displayed moderate to good activity against all the Gram-positive and Gram-negative bacterial strains with the MIC values ranging from 0.03 to 8.00 mg/ml. In general, *S. chamelaeagnea* exhibited the most favorable activity. The solvent extracts also exhibited promising activity against *M. tuberculosis* with the MIC values ranging between 0.10 and 0.50 mg/ml with *S. dolomitica, S. radula* and *S. verbenaca* displaying the best activity (MIC value: 0.10 mg/ml). The essential oils showed moderate activity against *B. cereus* and *K. pneumoniae*, while negligeable activity was observed against *E. coli* and *S. aureus* (MIC value > 12 mg/ml). The *in vitro* antibacterial activity of *S. chamelaeagnea* and *Leonotis leonurus* combined at various ratios showed in general synergistic interaction against the Gram-positive bacterial strains, while antagonistic and/or additive interactions were observed for the Gram-negative organisms. The antibacterial bio-assay guided fractionation of *S. chamelaeagnea* resulted in the isolation, identification and characterization of four compounds, namely carnosol, 7-*O*-methylepirosmanol, oleanolic acid and its isomer ursolic acid as the active principles against *S. aureus*. The *in vitro* antibacterial and antimycobacterial activities add scientific support to the use of *Salvia* species in traditional medicine against tuberculosis and a range of other bacterial infections.

9.1 Introduction

Micro-organisms are involved in the pathogenesis of many diseases and cause deterioration of a variety of products. Despite the progress in understanding the life cycle and control of many pathogens, nearly all the diseases affecting millions of people in developing countries are still caused by micro-organisms. This is mainly due to factors such as inadequate sanitation, poor hygiene and overcrowded living conditions (Wagstaff, 2002).

Tuberculosis (TB), a mycobacterial infection, remains a serious health problem in many regions of the world. It is estimated that a third of the world's population is infected with *Mycobacterium tuberculosis* and approximately 1.7 million people died of TB in 2003 (WHO, 2005b). In 2004, TB killed half a million people in Africa, mostly young men and women in their most productive years. Africa is the only continent where the TB rates are increasing. In just 15 years, overall rates have doubled, tripled in HIV endemic areas, and quadrupled in countries worst affected by HIV and TB (WHO, 2005b). The current threat to the success of TB treatment lies in the emergence of strains resistantance to the best antituberculosis drugs, namely isoniazid and rifampicin (Mativandlela *et al*., 2006) and the extreme drug-resistant TB strain that emerged in South Africa has drastically compromised the treatment programmes to eradicate TB. Common symptoms associated with TB include night sweats, fatigue, chest pains, loss of appetite resulting in weight loss (WHO, 2006a).

Infections associated with bacterial pathogens are among some of the conditions treated using traditional remedies in South Africa (Watt and Breyer-Brandwijk, 1962). In the past, the development of resistance by pathogens to many of the commonly used antimicrobial agents provided sufficient impetus for further attempts to search for new antimicrobial agents to combat infection (Grange and Davey, 1990). Sage is a potential source for new antimicrobial agents since it was used prior to the discovery of antimicrobial agents as a common component of herbal mixtures to treat the symptoms of tuberculosis and microbial infections (Watt and Breyer-Brandwijk, 1962).

The use of synergistic combinations is often needed in the treatment of serious infection and to reduce the risk of developing resistant strains. In traditional medicine, healers mostly rely on a combination of plants to treat diseases (Iwu, 1994). However, no *in vitro*

studies have as yet been reported on the effect of combining *Salvia* species with other plants against bacteria.

A survey of the literature revealed that *Salvia* species were usually used by Europeans in the Western Cape of South Africa with *Leonotis leonurus* (L.) R.Br. (Lamiaceae) to treat influenza, chest inflammation, pulmonary tuberculosis, skin diseases, colds and coughs (Watt and Breyer-Brandwijk, 1962). This information prompted an investigation into the *in vitro* antibacterial activity of *S. chamelaeagnea* and *L. leonurus* in combination.

The *in vitro* antibacterial and antimycobacterial screening provided the preliminary information for the selection of those active crude extracts of plants that were subjected to further chemical analysis.

The objectives of this study were to:

(i) investigate the antibacterial activity of the essential oils and solvent extracts of indigenous *Salvia* species,

(ii) evaluate the potential of the solvent extracts in inhibiting the growth of the tuberculosis pathogen,

(iii) evaluate the *in vitro* antibacterial activity of *S. chamelaeagnea* and *L. leonurus* in combination, and

(iv) isolate, identify and characterise the compounds exhibiting antibacterial activity from one of the active species.

9.2 Materials and methods

9.2.1 Reagents, chemicals and drugs

Tryptone soya agar (TSA), Tryptone soya broth (TSB) and ciprofloxacin were purchased from CA Milsch, while *p*-iodonitrotetrazolium chloride (INT), ursolic acid, oleanolic acid and rifampicin were from Sigma®. The BACTEC™ 12B media was purchased from Becton Dickinson. All solvents used were analytical grade from Rochelle Chemicals. The blood agar was obtained from the National Heath Laboratory Services (NHLS, Johannesburg).

9.2.2 Source and maintenance of micro-organisms

Four strains of bacteria were investigated and included *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae* (NTCC 9633) and *Escherichia coli* (ATCC 8739). The reference stock cultures were obtained from the NHLS (Johannesburg) and were maintained in the microbiology laboratory of the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg, South Africa. The maintenance was carried out using TSA prepared by dissolving 30 g in 750 ml of sterile water. The agar was autoclaved for 15 minutes at 121 ºC before use in order to sterilise the medium.

Mycobacterium tuberculosis H37Ra ATCC 25177 was used for the antimycobacterial experiments. The stock culture in glycerol was stored at -70 °C until experimentation. The stock was used to inoculate a 12B vial (Figure 9.1) and incubated at 37 °C. The 12B medium is an enriched Middlebrook 7H9 broth base containing ^{14}C-labeled palmitic acid for radiometric detection of mycobacterial growth.

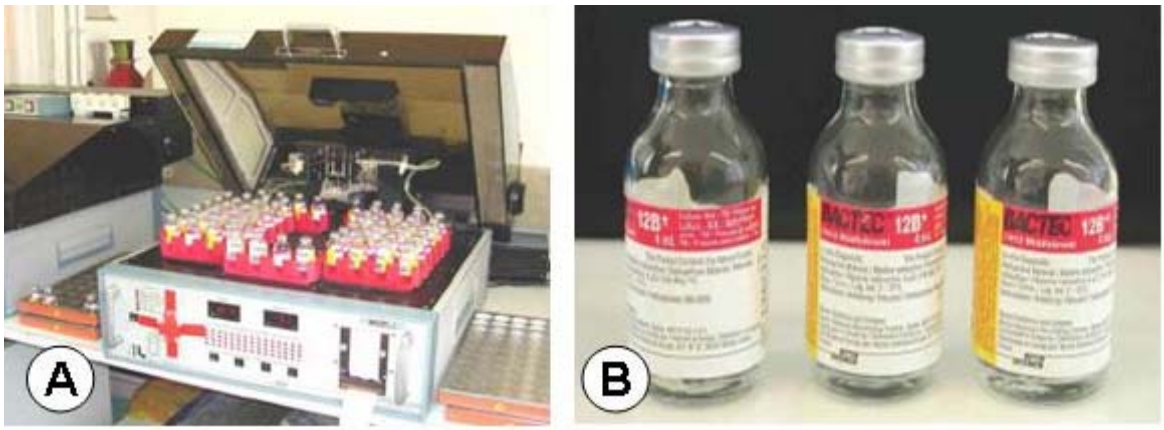


Figure 9.1 (A): BACTEC^{TM} 460 apparatus with racks containing inoculated vials; **(B):** the 12B vials containing the ^{14}C carbon substrate.

If micro-organisms are viable in the test sample, they utilize the ^{14}C-labeled substrate (palmitic acid) present in the medium and release $^{14}CO_2$ into the head space above the medium. When tested on the BACTEC^{TM} 460 instrument, the gas is aspirated from the vial and the $^{14}CO_2$ radio-activity is measured and displayed as a growth index (GI) ranging from 0 to 999 (Middlebrook *et al.,* 1977). The daily increase in the GI is directly proportional to the rate and amount of growth in the medium.

9.2.3 Sample preparation

Stock solutions of both plant extract (64 mg/ml) and essential oil (128 mg/ml) for the were prepared with acetone for the antibacterial study. For the combination experiment, the concentration of each plant was first carried out at 64 mg/ml and various ratios using the stock solution prepared. The stock solution used in the antimycobacterial activity testing was prepared at a concentration of 42 mg/ml with 50% methanol. The animycobacterial activity of the essential oils was not performed because the quantity of oil was not sufficient.

9.2.4 Determination of the antibacterial and antimycobacterial activities

9.2.4.1 Antibacterial activity: micro-dilution method

The *in vitro* antibacterial activity was evaluated by a micro-dilution assay (Eloff, 1998). Dilutions were prepared in a sterile, flat-bottom, 96-well microtitre plate. In a 96-well plate, 100 µl of sterile water was plated out in all the wells. In the first row, 100 µl (stock solution) of plant extract/essential oil was added to the well and 100 µl transferred to the next row after thorough mixing with the water in the 96-well microtitre plate using a multichannel pipette. This dilution series is prepared from the top to the bottom of the plate until the last row where 100 µl from each well was discarded. The bacterial suspension (100 µl), yielding an inoculum size of approximately 1 x 10^6 CFU/ml was added to all the wells. The final concentration in the wells ranged from 16.00 to 0.13 mg/ml (extract) and from 32.00 to 0.50 mg/ml (essential oil). Further serial dilutions were made where necessary. The plate was then sealed and incubated at 37 ºC overnight. After incubation, 50 µl of 0.04% (w/v) INT was added directly into each well and the plate left at room temperature for six hours before the readings were taken. The method is based on the principle that the colourless tetrazolium compound (INT) acts as an electron acceptor and is reduced to a coloured product by biologically active organisms (Eloff, 1998). Sterile water was used as a negative control, while ciprofloxacin was used as the positive antibacterial control. The final concentration (3%) of acetone in the well did not adversely affect the bacterial growth.

The minimum inhibitory concentration (MIC) value was determined as the lowest concentration of sample required to inhibit the growth of test organisms. In the microtitre plate, the MIC can be seen as the lowest concentration where the clear well is observed

(Figure 9.2). In some cases, the colour of the extract prevents a good visualisation of the bacterial growth/inhibition. Thus, the agar plate was prepared in order to double check whether there was effectively no bacterial growth.

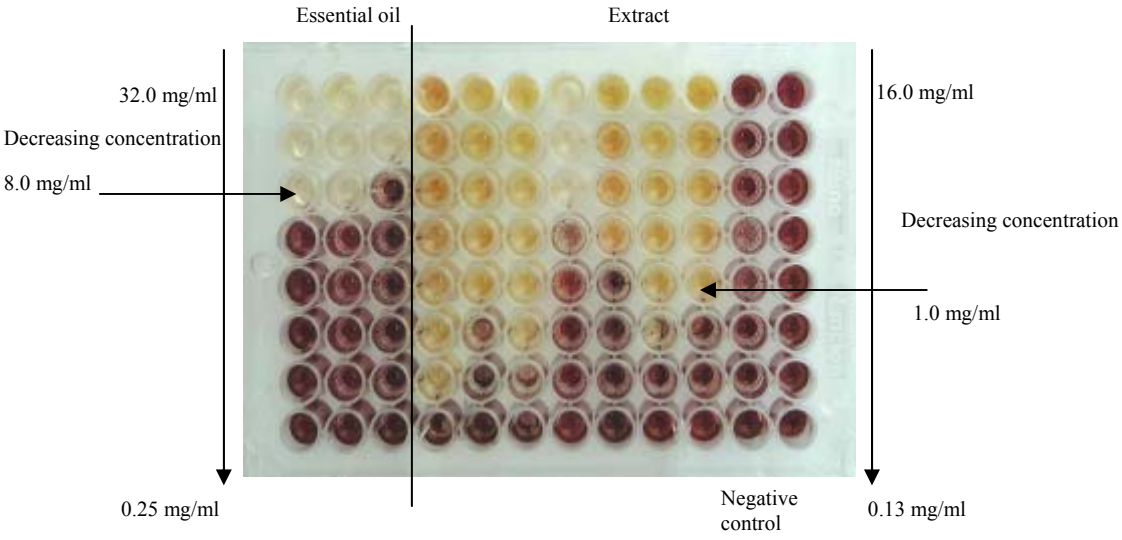


Figure 9.2 An example of the determination of the MIC value using a 96-well microtitre plate. The first clear well from the bottom indicates the MIC value, i.e. the concentration at which no bacterial growth is obtained and the purple well indicates bacterial growth.

9.2.4.2 Combination study of *S. chamelaeagnea* and *L. leonurus*

To study the combined antibacterial activity of *S. chamelaeagnea* and *L. leonurus* (methanol:chloroform, 1:1 v/v), ten combinations at various ratios were prepared utilising extracts with an initial concentration of 64 mg/ml in acetone. The following ratios of *S. chamelaeagnea* and *L. leonurus* were considered, respectively: 10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 3:7; 2:8; 1:9 and 0:10. A volume of 100 µl of different combinations was introduced into a 96-well microtitre plate containing 100 µl of water and the experiment was then carried out as described in Section 9.2.4.1.

In order to analyse the outcome of the combination study, the sum of fractional inhibitory concentration of *S. chamelaeagnea* and *L. leonurus* (sum FIC_{SL}) was mathematically determined (Eq. 9.1) (Berenbaum, 1978). All ratios were presented graphically by construction of isobolograms as described by Canfield *et al*. (1995). The data points for each pathogen were obtained for various ratios by plotting the MIC_S in combination divided by the MIC_S alone on the X axis versus the MIC_L in combination divided by the

MIC_L alone (on the Y axis). Based on the sum FIC_{SL} values, values < 1 indicate synergistic interactions, values > 1 show antagonistic interactions, while values ≈ 1 represent additive interactions. On the basis of the isobolograms, data points above the straight line have antagonistic interactions, those below have synergistic interactions and those in the vicinity of the line have additive interactions.

$$\text{sum } FIC_{SL} = \frac{MIC_S \text{ in combination}}{MIC_S \text{ alone}} + \frac{MIC_L \text{ in combination}}{MIC_L \text{ alone}} \quad \text{(Eq 9.1)}$$

Where: FIC_{SL} = Sum of the fractional inhibitory concentration of *S. chamelaeagnea* and *L. leonurus.*

MIC_S = Minimum inhibitory concentration of *S. chamelaeagnea.*

MIC_L = Minimum inhibitory concentration of *L. leonurus.*

For each analysis, samples were performed in duplicate and at least three experiments were carried out per test sample.

9.2.4.3 Bio-assay on *M. tuberculosis*: BACTEC™ method

The susceptibility testing of the solvent extracts against *M. tuberculosis* was conducted using the radiometric BACTEC™ technique as described by Siddiqi *et al.* (1981), Roberts *et al*. (1983) and Scarparo *et al.* (2002). This method, despite the relatively high cost of the 12B culture medium, was preferred to the agar method for plant extract susceptibility testing because the radiometric method allows greater cell-to-drug interaction in the liquid medium as described by Lall and Meyer (1999).

The 12B vials were inoculated with *M. tuberculosis* from the stock solution, incubated at 37 °C and changes in the GI were recorded daily until a value of 999 was obtained. The mycobacterial suspension was adjusted to approximately match that of a McFarland No. 1 turbidity standard (Lall and Meyer, 1999). Thereafter, a 12B vial was inoculated with 100 µl of the mycobacterial suspension and incubated at 37 °C. When the growth in this vial reached 400-500, the 7H9 broth culture was used undiluted to inoculate a set of 12B vials (100 µl per vial), yielding approximately 1 x 10^4-1 x 10^5 CFU/ml (Heifets *et al*., 1985). Before the extracts were added to the vials, each sample was filtered through a 0.22 µm Whatman® filter unit to prevent any contamination. The final extract concentration of 1, 0.5 and 0.1 mg/ml were initially prepared in 50% methanol. Vials were incubated at 37 °C

and visually monitored every day for turbidity as an indicator of contamination and any suspected contaminants were confirmed by culturing on 2% blood agar plates (NHLS, Greenpoint) made with Mueller Hinton (Oxoid) and 2% blood. Two plant extract-free vials were used as controls. One vial was inoculated with the organism in the same way as the vial containing plant extracts and the other was inoculated with a 1:100 dilution of the inoculum obtained by adding 0.10 ml of inoculum to 9.90 ml of 12B medium. A methanol control at 2.38%, equivalent to the maximum solvent concentration in the 12B test samples, had no negative impact on the normal growth of *M. tuberculosis*.

The radiometric susceptibility method indicates that the concentration of the extract that results in a daily increase and final ΔGI reading lower than in the control (1:100) can be considered the concentration at which more than 99% of the bacterial population is inhibited. Rifampicin, a standard antituberculosis drug, was used as positive control at a final concentration of 2.00 μg/ml. The difference in the GI values for the last two days is designated as ΔGI. The GI readings of the plant extracts were compared with the control vial containing 1:100 dilution of the inoculum. Once the ΔGI of the control reached 30, the results were interpreted by comparing the increase with that in the test vial. The following formula was used to interpret the results: ΔGI control > ΔGI drug = susceptible; ΔGI control < ΔGI drug = resistant and the MIC value was defined as the lowest concentration of drug inhibiting more than 99% of the *M. tuberculosis* population. The final MIC value was confirmed by repeat testing.

9.2.5 Isolation of antibacterial active compounds from *S. chamelaeagnea*

S. chamelaeagnea which displayed the most favourable antibacterial activity was selected to isolate the active principles. Combinations of various methods were used in isolation, identification and characterization procedures.

9.2.5.1 Extraction

Dried aerial parts (423.4 g) collected at the Kirstenbosch Botanical Garden were powdered and extracted with methanol:chloroform (1:1, v/v) and the residue was evaporated under reduced pressure to yield 19.6 g.

9.2.5.2 Column chromatography

The residue was mixed with a small quantity of Silica gel 60 (0.063-0.200 mm/70-230 mesh ASTM, Macherey-Nagel), dried at 60 °C in an oven, reduced to a fine powder and added to the top of the column. The column (4 cm x 60 cm) was eluted successively with hexane:dichloromethane (9:1), then dichloromethane:methanol (6:1) followed by 100% methanol. The collection of these crude fractions were based on colour change. The fractions were collected in 250 ml conical flasks and similar spots on TLC were combined to yield five main fractions (A-E).

9.2.5.3 Thin layer chromatography and bio-autographic assay

A direct antibacterial bio-assay (Dilika and Meyer, 1996) on a TLC plate was employed using the agar-overlay method with *S. aureus* as the test organism. *S. aureus* was selected because it is amongst the most difficult bacterial strains to combat with classic antibacterial agents (Tomás-Barberán *et al*., 1990). Each of the five fractions (Section 9.2.5.2) were spotted on a TLC plate (Alugram® Sil G/UV_{254}, 0.200 mm) developed with toluene:dioxan:acetic acid glacial (95:25:5) as eluent. The dried TLC plate was then sterilised under UV light (254 nm) for one hour before being placed in a petri dish containing the solidified agar. The UV light was used to sterilise the dried extract instead of autoclaving in order to avoid denaturing the active constituents (Okoli and Iroegbu, 2004). The plate was then overlayed with *S. aureus* culture (1 x 10^6 CFU/ml) in TSA and left to solidify. After one hour at 4 °C (to allow the compounds on the TLC to diffuse), the plate was removed and incubated at 37 °C for 24 hours. At the end of the incubation period, the TLC plate was sprayed with 0.04% (w/v) aqueous solution of INT to visualise the inhibition zone. The inhibition zone was indicated by the clear zone on the purple background (Figure 9.3). Only fraction D (Figure 9.3) was active and thus selected for further analysis.

9.2.5.4 Bioactivity-guided fractionation and isolation

Fraction D was evaporated under vacuum to yield 6.21 g. This fraction was rechromatographed on a small column (2.5 cm x 45 cm) using hexane:ethyl acetate: acetic acid (12:3:0.02) as eluent. A total of 322 sub-fractions in 3 ml test tubes were collected. The fractions were spotted on the TLC plate and developed in toluene:dioxan:acetic acid glacial (95:25:5) and similar fractions were combined and subjected to a bio-autographic

assay on TLC to detect fraction(s) containing the active compound(s). The eluted fractions were monitored on a TLC plate developed with the same solvent system.

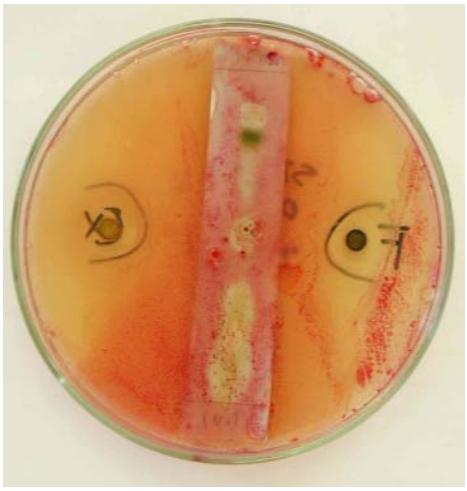


Figure 9.3 Bio-autographic assay of *Salvia chamelaeagnea* against *Staphylococcus aureus*: Inhibition zones are shown by clear spots on the purple background.

Pooled fractions were concentrated with a rotary evaporator and rechromatographed using the same column with hexane:ethyl acetate:acetic acid (12:2:0.01) as the solvent system. The column chromatography of the active fraction was finally carried out with toluene:ethyl acetate (10:3) and 293 sub-fractions were obtained. Throughout the isolation procedure, crystals formed were further purified by recrystalisation from acetone. The bioactivity-guided methodology is summarized in Figure 9.4.

9.2.5.5 Identification and elucidation of isolated compounds

The isolated compounds were structurally characterised using NMR and MS and data compared to those found in the literature [**1** and **2** (Urones *et al*., 1998; Ahmed *et al*., 2006), **3** and **4** (Mahato and Kundu, 1994)].

(i) Nuclear magnetic resonance (NMR)

^{1}H, ^{13}C, HSQC, HMBC, DEPT, NOESY and COSY NMR data of compounds **1**, **2**, **3** and **4** were recorded on a Varian Inova 2000 300 MH_Z spectrometer. All spectra were recorded at 25 °C in deuterated chloroform and the chemical shifts were recorded in ppm referenced to TMS.

(ii) Mass spectrometry

Electron ionization MS of the isolated compounds was performed by direct inlet at 70 eV on the GC-MS QP 2010 GC coupled to MS.

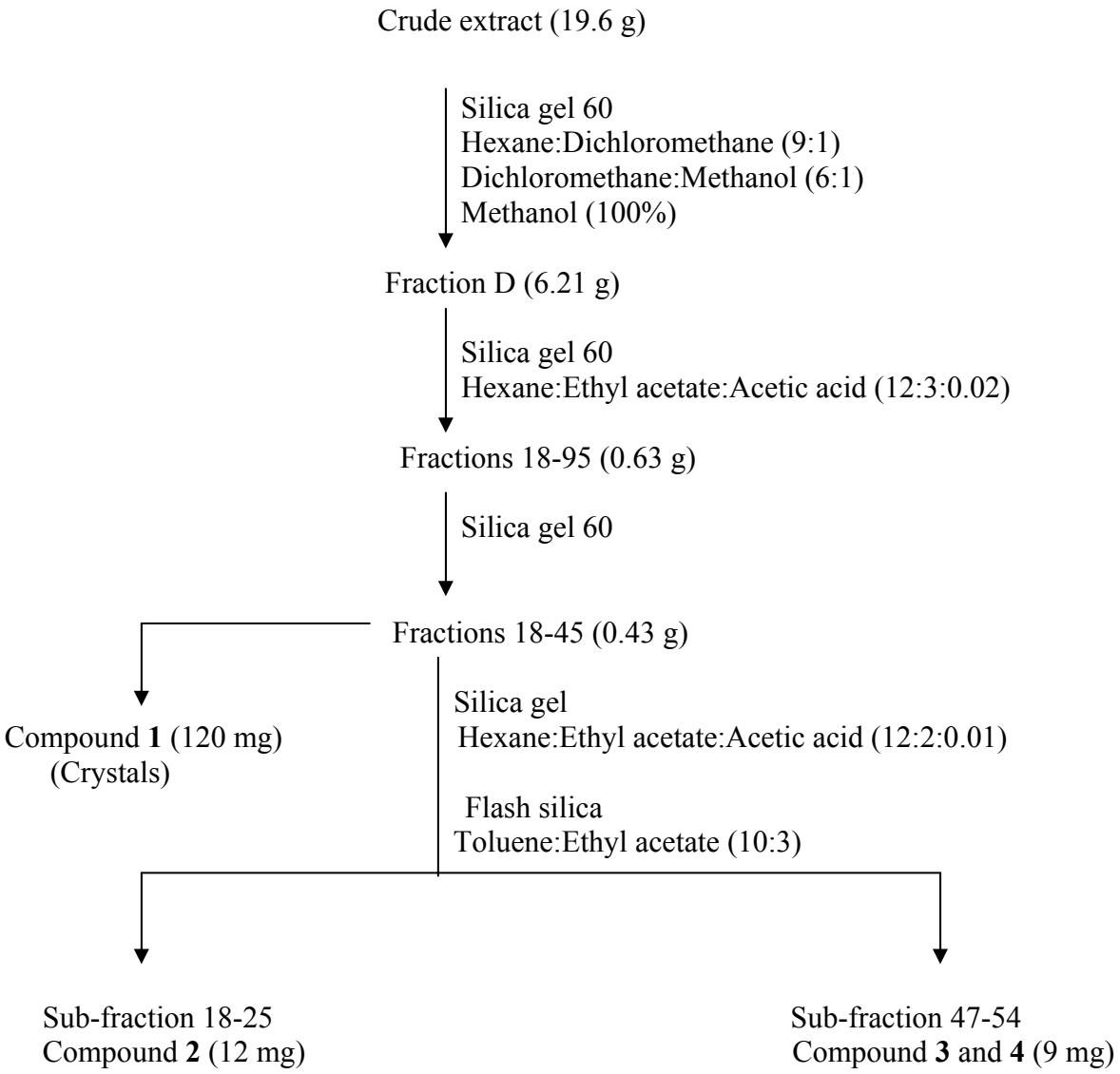


Figure 9.4 Bio-assay guided fractionation for compounds isolated from *S. chamelaeagnea*.

9.3 Results

9.3.1 Antibacterial activity

The solvent extracts and essential oils affected the test organisms to different degrees and the results of the antibacterial activity showed that the solvent extracts exhibited better antibacterial activity than the essential oils (Table 9.1).

Table 9.1 The MIC values (mg/ml) of the solvent extracts and essential oils of *Salvia* species against Gram-positive and Gram-negative bacteria (n = 3).

Species/Isolated	*E. coli* (ATCC 8739)		*K. pneumoniae* (NCTC 9633)		*B. cereus* (ATCC 1177)		*S. aureus* (ATCC 25923)	
compound/Controls	Ext	EO	Ext	EO	Ext	EO	Ext	EO
S. africana-caerulea	4.00	16.00	4.67	8.00	6.00	0.75	3.00	4.0
S. africana-lutea	3.00	> 32.00	3.00	8.00	0.75	2.33	0.75	8.00
S. albicaulis	4.00	> 32.00	4.67	8.00	1.00	2.33	1.00	8.00
S. aurita	4.00	nd	4.000	nd	0.25	nd	0.05	nd
S. chamelaeagnea	1.00	> 32.00	3.0	12.00	0.03	2.00	0.06	> 32.00
S. disermas	4.00	nd	2.00	nd	3.00	nd	0.75	nd
S. dolomitica	3.33	> 32.00	2.00	8.00	0.75	9.33	0.03	12.00
S. garipensis	1.88	nd	2.81	nd	0.75	nd	4.00	nd
S. lanceolata	4.00	32.00	2.00	16.00	3.00	1.00	2.00	8.00
S. muirii	2.00	> 32.00	3.00	6.00	0.25	9.33	0.36	> 32.00
S. radula	> 16.00	nd	4.00	nd	1.00	nd	0.06	nd
S. repens	2.00	nd	3.00	nd	0.03	nd	0.25	nd
S. runcinata	2.00	> 32.00	4.00	> 32.00	0.03	2.00	0.25	> 32.00
S. schlechteri	4.00	nd	3.00	nd	0.75	nd	0.25	nd
S. stenophylla	4.00	> 32.00	2.00	8.00	0.03	1.50	0.06	> 32.00
S. verbenaca	8.00	nd	2.00	nd	2.00	nd	3.0	nd
Carnosol	2.00		2.00		0.02		0.03	
Oleonolic acid	3.41		1.71		1.28		3.75	
Ursolic acid	2.50		3.75		7.32×10^{-3}		0.01	
7-*O*-Methyl-*epi*-rosmanol	nd		nd		0.01		0.02	
Ciprofloxacin	4.00×10^{-5}		1.60×10^{-4}		4.10×10^{-5}		3.1×10^{-4}	

nd: not determined, essrential oil insufficient or not available; EO: essential oil; Ext: solvent extract.

Nearly all the solvent extracts evaluated for antibacterial activity were active against the strains used. While essential oils exhibited moderate activity against *B. cereus* and *K. pneumoniae* and were almost completely inactive against *E. coli* and, to a certain degree, *S. aureus* (Table 9.1).

Among the plants screened, only the solvent extract of *S. radula* failed to inhibit the growth of *E. coli,* although displaying good activity against other pathogens. The best activity of the solvent extracts against *B. cereus* was obtained with *S. chamelaeagnea*, *S. radula*, *S. runcinata* and *S. stenophylla* (MIC value: 0.03 mg/ml), while *S. africana-caerulea* displayed the lowest activity (MIC value: 6.00 mg/ml). The best activity against *S. aureus* was obtained with *S. dolomitica* (MIC value: 0.03 mg/ml). Five species, namely *S. disermas*, *S. dolomitica*, *S. lanceolata*, *S. stenophylla* and *S. verbenaca* were the most active against *K. pneumoniae* (MIC value: 2.00 mg/ml). Finally, the best activity against the Gram-negative *E. coli* was obtained with the solvent extract of *S. chamelaeagnea* (MIC value: 1.00 mg/ml) and *S. muirii* (MIC value: 1.88 mg/ml). It was interesting to observe that the essential oil of *S. africana-caerulea* was more active than its solvent extract against *B. cereus*.

The isobolograms of *S. chamelaeagnea* and *L. leonurus* at various ratios against the four pathogens are shown in Figure 9.5. Synergistic interactions were obtained against Gram-positive bacteria for nearly all ratios. Only the ratio 2:8 of *S. chamelaeagnea* and *L. leonurus* showed antagonistic interactions against *S. aureus* (data point corresponding to 2.50 and 1.24, cannot be seen on the graph, but on Table 9.2 as 3.74). Mostly antagonistic or additive interactions were observed with Gram-negative bacteria (Figure 9.5). However, ratios 5:5, 3:7 and 2:8 showed synergistic interactions against *E. coli*, while ratios 7:3 and 5:5 were also synergistic against *K. pneumoniae*.

The sum of fractional inhibitory concentration values of *S. chamelaeagnea* and *L. leonurus* (sum FIC_{SL}) are displayed in Table 9.2. It can be seen that the response of the combination between the two plants varied with ratios and the specific test organism. Similarly, only ratio 2:8 (FIC_{SL} = 3.74) showed antagonistic interactions against the Gram-positive bacteria, while all the remaining ratios were synergistic ($FIC_{SL} < 1$).

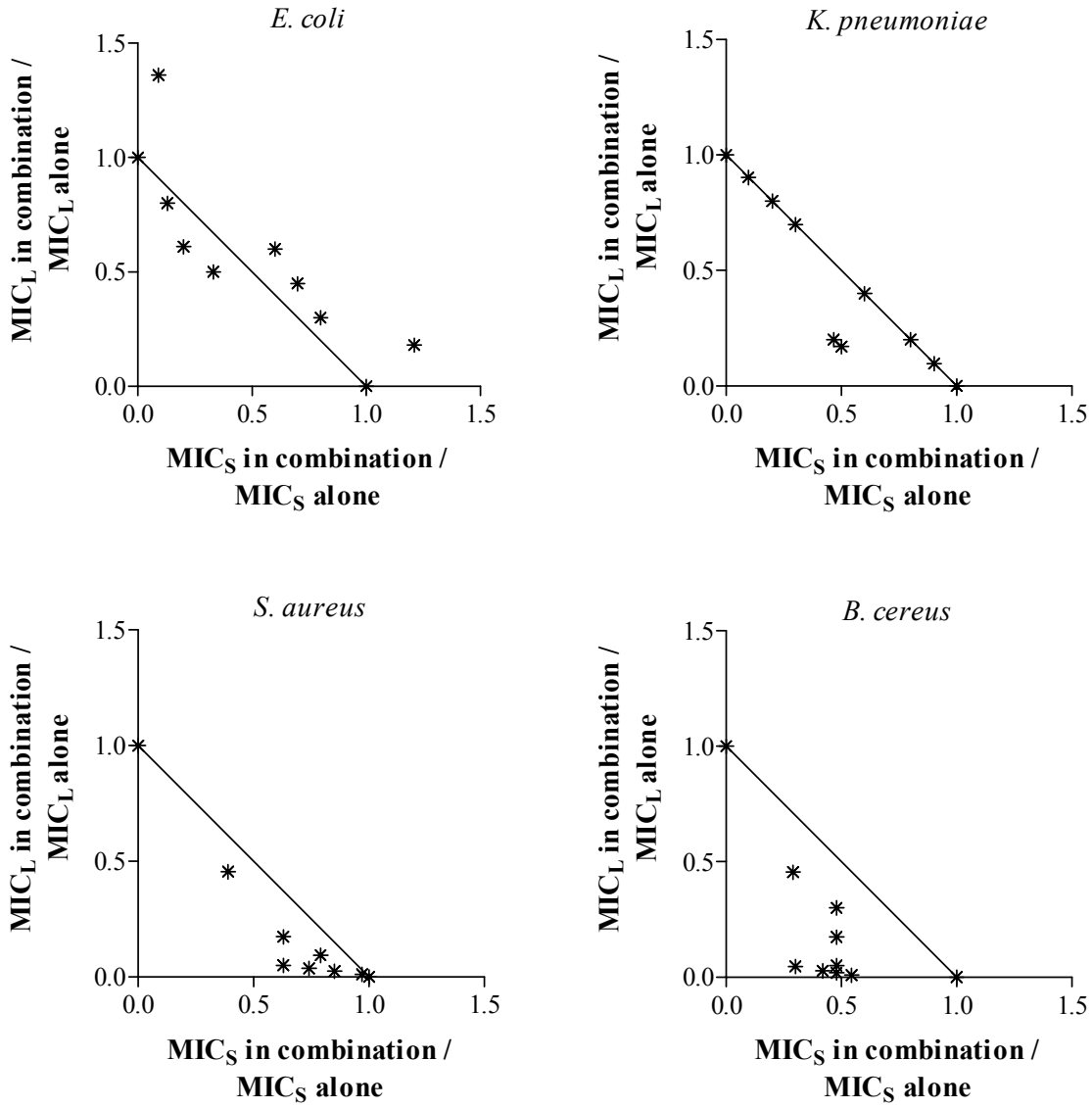


Figure 9.5 Isobolograms constructed from combination study involving *Salvia chamelaeagnea* and *Leonotis leonurus* on four bacterial pathogens.

The combination of *S. chamelaeagnea* and *L. leonurus* at various ratios showed synergistic interactions at only three ratios (5:5, 3:7 and 2:8) against *E. coli,* while against *K. pneumoniae*, the synergistic interactions were obtained at only two ratios (7:3 and 5:5) (sum $FIC_{SL} < 1$, Table 9.2).

This study revealed that Gram-positive bacteria were more sensitive compared to Gram-negative bacteria. *E. coli* was in general the most resistant strain especially against the essential oils with all MIC values obtained greater than 32 mg/ml.

Table 9.2 The sum of the fractional inhibitory concentration (sum FIC_{SL}) values of *Salvia chamelaeagnea* and *Leonotis leonurus* using various combinations ratios.

Ratios	Pathogens			
	E. coli **(ATCC 8739)**	***K. pneumoniae*** **(NCTC 9633)**	***B. cereus*** **(ATCC 11778)**	***S. aureus*** **(ATCC 11778)**
10:0	1.00	1.00	1.00	1.00
9:1	1.39	1.00	0.55	0.99
8:2	1.10	1.00	0.50	0.87
7:3	1.15	0.67	0.45	0.78
6:4	1.20	1.00	0.53	0.68
5:5	0.83	0.67	0.35	0.89
3:7	0.81	1.00	0.66	0.81
2:8	0.93	1.00	0.78	3.74
1:9	1.45	1.00	0.75	0.84
0:10	1.00	1.00	1.00	1.00

Values < 1 denote synergism interactions; values > 1 denote antagonism interactions and values ≈ 1 denote additive interactions.

9.3.2 Antimycobacterial activity

Mycobacterium tuberculosis was sensitive to all the solvent extracts at a concentration of 1.00 mg/ml (Table 9.3). Three species, namely *S. dolomitica*, *S. radula* and *S. verbenaca* inhibited mycobacterial growth at a concentration of 0.10 mg/ml. Therefore, further dilutions yielded a MIC value of 0.10 mg/ ml for all three extracts (Table 9.3).

Since the three species exhibited the same activity, the GI was plotted at 0.10 and 0.05 mg/ml and the results are depicted in Figure 9.6. It is clear that *S. verbenaca* was the most active extract because its GI readings at 0.10 and 0.05 mg/ml were low compared to that of *S. radula* and *S. dolomitica* (Figure 9.6) when tested at the same concentration.

It was noted that these three species also exhibited good antibacterial activity against *S. aureus* and *B. cereus* (Table 9.1).

Table 9.3 The effect of plant extracts on the growth of *Mycobacterium tuberculosis* using the radiometric method.

Plant species	ΔGI values (mg/ml)			MIC values (mg/ml)
	1.00	0.50	0.10	
S. africana-caerulea	0 (S)	-3 (S)	73 (R)	0.50
S. africana-lutea	0 (S)	0 (S)	50 (R)	0.50
S. albicaulis	0 (S)	0 (S)	41 (R)	0.50
S. aurita	0 (S)	4 (S)	33 (R)	0.50
S. chamelaeagnea	(S)	1 (S)	37 (R)	0.50
S. disermas	0 (S)	18 (S)	520 (R)	0.50
S. dolomitica	2 (S)	-1 (S)	11 (S)	0.10
S. lanceolata	0 (S)	-1 (S)	40 (R)	0.50
S. muirii	0 (S)	7 (S)	33 (R)	0.50
S. radula	-1 (S)	4 (S)	10 (S)	0.10
S. repens	0 (S)	2 (S)	48 (R)	0.50
S. runcinata	0 (S)	4 (S)	40 (R)	0.50
S. schlechteri	0 (S)	19 (S)	38 (R)	0.50
S. stenophylla	0 (S)	8 (S)	38 (R)	0.50
S. verbenaca	-2 (S)	3 (S)	5 (S)	0.10
Rifampicin	0 (S)	0 (S)	0 (S)	0.002

S, sensitive; R, resistant, ΔGI values of the control vials were 29, 29 and 28 at 1, 0.5 and 0.1 mg/ml, respectively. For the sample to be active against *M. tuberculosis*, ΔGI must be less or equal to ΔGI of the control vial. A rifampicin control vial (2 µg/ml) was included in each experiment.

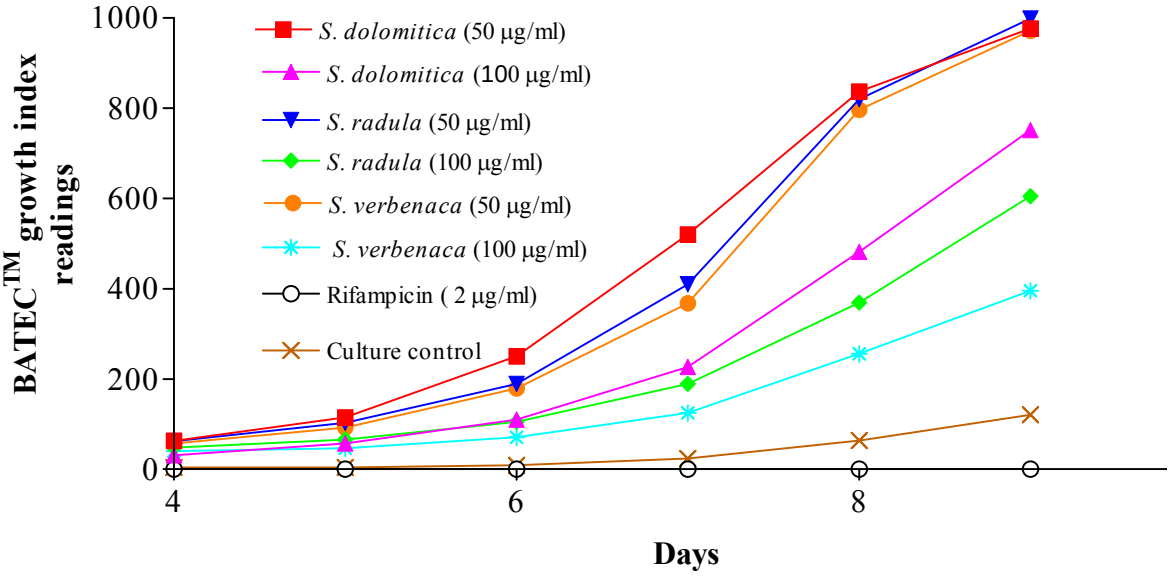


Figure 9.6 Growth index of three *Salvia* species tested at two different concentrations against *Mycobacterium tuberculosis*.

9.3.3 Elucidation and identification of the isolated compound from *S. chamelaeagnea*

The ^{1}H-NMR and ^{13}C-NMR data of the four isolated compounds are presented in Table 9.4 to Table 9.6. The ^{1}H, ^{13}C spectra are shown in Appendix E. Based on the ^{1}H-NMR , ^{13}C-NMR, DEPT, COSY spectra and comparison with the data obtained in the literature, compound **1** was identified as 2H-9,4a-(epoxymethano) phenanthren-12-one,1,3,4,9,10,10a-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylmethylethyl)-4aR,9S,10aS)-(9CI) (carnosol) and established to have a molecular formula of $C_{20}H_{26}O_4$. Compound **2** was a diterpene identified as the 7-*O*-methylepirosmanol and established to have a molecular formula of $C_{21}H_{28}O_5$. Compound **3** and **4** (triperpenoid) were obtained as co-eluting mixture and identified as [(3β)-3-hydroxyurs-12-en-28-oic acid], commonly known as ursolic acid and the chemical formula is ($C_{30}H_{48}O_3$), while compound **4,** which is an isomer of compound **3,** was characterized as [(3β)-3-hydroxyolean-12-en-28-oic acid] commonly known as oleanolic acid with the chemical formula ($C_{30}H_{48}O_3$). The UV and chromatographic data of the isolated compounds are shown in Figure 5.1 and Table 5.1, while the absolute stereochemistries of the isolated compounds are presented in Figure 9.7.

Table 9.4 NMR data (in CD_3OD) of compound **1** isolated from *Salvia chamelaeagnea*.

Position	δ_C	δ_H	Correlations observed in HMBC	NOESY
1	29.0	2.82-2.88, dt	C-2, 10, 20	
2	18.9	1.64, t		
3	41.0	1.58-1.62, m		
4	34.3			
5	45.9	1.74, m	C-9, 10, 18, 19, 20	
6	29.7	2.25, m	C-4, 7, 8, C-5	
7	78.6	5.5, s		
8	132.2			
9	121.8			
10	48.6			
11	143.6	4.90, s		
12	143.1	4.90, s		
13	134.9			
14	111.4	6.74, s	C-7, 9, 12, 15	H-7
15	26.8	3.30, m	C-12, 13, 14, 16, 17	
16	22.1	1.22-1.28, m	C-15, 17	
17	22.1	1.22-1.28, m	C-15, 16	
18	31.0	0.80, s	C-3, 4, 5, 18	
19	18.9	0.80, s	C-3, 4, 5, 19	
20	178.2			

CD_3OD: methanol: HMBC: heteronuclear multiple bond correlation, NOESY: nuclear overhauser effect spectroscopy, singlet, dt: double of triplets, t: triplet, m: multiplet, δ_C: carbon shift, δ_H: proton shift.

Table 9.5 NMR data (in $CDCl_3$) of compound **2** isolated from *Salvia chamelaeagnea*.

Carbon	δ_C	δ_H
1	26.9	
2	18.8	
3	37.7	
4	31.7	
5	55.5	
6	74.2	4.35, d, *J* = 2.8 Hz
7	78.0	4.85, d, *J* = 2.8 Hz
8	125.7	
9	124.8	
10	47.8	
11	143.9	
12	141.2	
13	135.3	
14	119.7	6.84, s
15	27.1	
16	22.0	1.11, d
17	22.5	1.13, d
18	31.5	0.91, 18
19	21.8	0.87, 19
20	179.4	
OMe	56.0	3.50, s

$CDCl_3$: chloroform: δ_C: carbon shift, δ_H: proton shift, singlet, d: doublet, *J*: coupling constant, Hz: hertz.

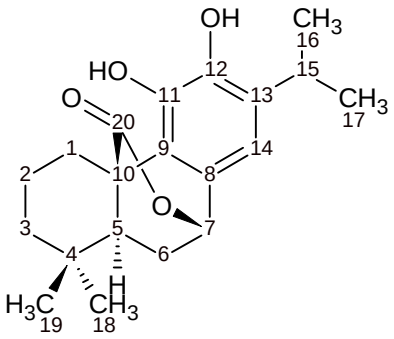
Table 9.6 NMR data (in $CDCl_3$) of the mixture of two triterpenoids: oleanolic and ursolic acid isolated from *Salvia chamelaeagnea.*

δ_C **Mixture**	δ_C **Oleanolic acid (**Mahato and Kundu, 1994)	δ_C **Ursolic acid (**Mahato and Kundu, 1994)
180.7 (s)	181.0 (s, C-28)	
180.5 (s),		180.4 (C-28)
143.7 (s)	143.4 (s, C-13)	
138.0 (s)		148.1 (s, C-13)
125.3 (d)		125.5 (d, C-12)
122.1 (d)	122.1 (d, C-12)	
78.7 (d) (2C)	78.7 (d, C-3)	78.9 (d, C-3)
55.1 (d) (2C)	55.2 (d, C-5)	55.1 (d, C-5)
52.7 (d)		52.7 (d, C-18)
47.7 (s)		47.7 (s, C-17)
47.5 (d)	47.6 (d, C-9)	
47.4 (d)		47.5 (d, C-9)
46.2 (s)	46.6 (s, C-17)	
45.8 (t)	45.8 (t, C-19)	
41.9 (s)		42.0 (s, C-14)
41.6 (s)	41.6 (s, C-14)	
41.0 (d)	41.3 (d, C-18)	
39.3 (s)	39.3 (d, C-8)	39.4 (d, C-8)
39.0 (s)	38.7 (s, C-4)	
38.9 (d)		39.0 (d, C-19)
38.7 (d)		38.6 (d, C-20)
38.52 (t)		38.8 (t, C-1)
38.49 (s)		38.6 (s, C-4)
38.3 (t)	38.5 (t, C-1)	
36.8 (s)	37.0 (s, C-10)	
36.7 (s)		37.0 (s, C-10)
36.6 (t)		36.7 (t, C-22)
33.7 (t)	33.8 (t, C-21)	
32.8 (q)	33.1 (q, C-29)	

δ_C Mixture	δ_C Oleanolic acid (Mahato and Kundu, 1994)	δ_C Ursolic acid (Mahato and Kundu, 1994)
32.8 (t)		33.0 (t, C-7)
32.6 (t)	32.8 (t, C-22)	
32.4 (t)	32.6 (t, C-7)	
30.5 (t)	30.6 (C-20)	30.6 (t, C-21)
27.84 (q)	28.1 (q, C-23)	28.2 (q, C-23)
27.80 (q)		28.0 (t, C-15)
27.5 (t)		27.9 (t, C-2)
26.7 (t)	27.7 (t, C-15)	
26.6 (t)	27.4 (t, C-2)	
25.6 (q)	26.0 (q, C-27)	
24.0 (t)		24.2 (t, C-16)
23.3 (q)	23.6 (q, C-30)	
23.2 (t)	23.4 (t, C-16)	
23.1 (t)		23.3 (t, C-11)
22.9 (t)	23.1 (t, C-11)	
20.9 (q)		21.1 (q, C-30)
18.5 (t) (2C)	18.3 (t, C-6)	18.2 (t, C-6)
16.8 (q)		16.9 (q, C-6, 27)
16.7 (q)	16.8 (q, C-26)	
16.6 (q)		16.8 (q, C-29)
15.40 (q)		15.7 (q, C-25)
15.35 (q)	15.6 (q, C-24)	
15.2 (q)		15.4 (q, C-24)
15.1 (q)	15.3 (q, C-25)	

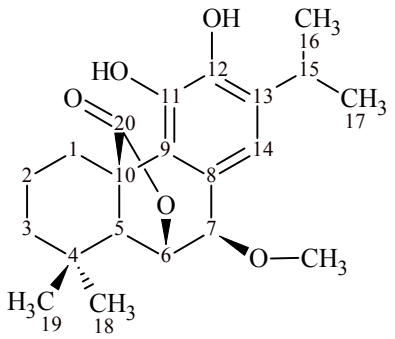
δ_H 5.21 (1H, br. s, H-12), 5.18 (1H, br. s, H-12), 3.14 (2H, t, J = 7.7 Hz, H-3).

$CDCL_3$: chloroform, s: singlet, d: doublet, t: triplet, q: quartet, δ_H: proton shift, J: coupling constant, Hz: hertz.



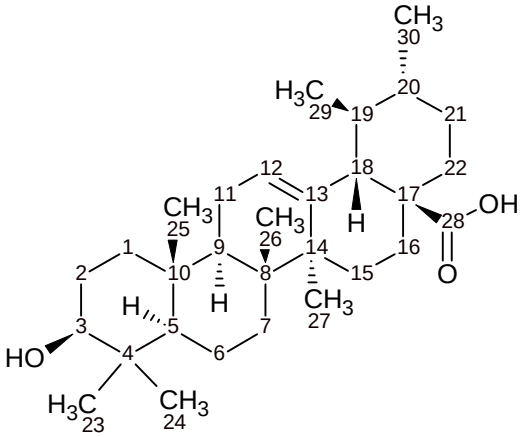
(1)

2H-9,4a-(epoxymethano)phenanthren-12-one, 1,3,4,9,10,10a-hexahydro-5,6-dihydroxy-1,1-dimethyl-7-(1-methylethyl)-4aR,9S,10aS)-(9CI) ($C_{20}H_{26}O_4$) (Carnosol)



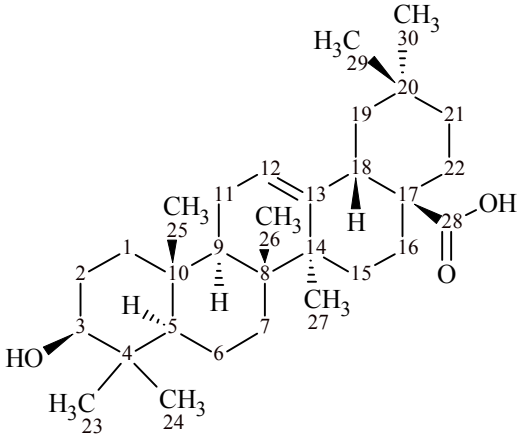
(2)

7-*O*-Methylepirosmanol ($C_{21}H_{28}O_5$)



(3)

(3β)-3-hydroxyurs-12-en-28-oic acid ($C_{30}H_{48}O_3$) (Ursolic acid)



(4)

(3β)-3-hydroxyolean-12-en-28-oic acid ($C_{30}H_{48}O_3$) (Oleanolic acid)

Figure 9.7 Structures of the isolated compounds with antibacterial activity from *Salvia chamelaeagnea*.

9.4 Discussion

The solvent extract displayed good to moderate antibacterial activity as shown by the low MIC values (Table 9.1). According to Fabry *et al*. (1998), the solvent extracts of plants with MIC values around 8.00 mg/ml may have some antimicrobial activity. The values obtained in this study were less than 8.00 mg/ml against all the pathogens tested and the

species studied can therefore be considered to display appreciable activity. More precisely, species such *S. dolomitica*, *S. radula* and *S. verbenaca* displayed good antibacterial and antimycobacterial activity with MIC values less than 3 mg/ml against *B. cereus* and *S. aureus* and MIC values of 0.10 mg/ml against *M. tuberculosis*, respectively (Table 9.1 and 9.3). The current results are consistent with the pattern for the *in vitro* activity emerging from other studies. The antibacterial activity of exotic *Salvia* species is reported in the literature (Lee *et al*., 1999; Ulubelen *et al*., 2001; Tepe *et al*., 2004) although the MIC values, the methodology used and pathogens differed. Variations reported between antimicrobial data are frequent and this can be explained by the difficulty to cultivate micro-organisms under the same test conditions, due to species-specific requirements (e.g. incubation temperature, growth medium and atmosphere). A further explanation for the differences in antibacterial activity data is the different sensitivity of strains, the mode and choice of solvent extraction and variation in the composition of the extracts.

Indigenous *Salvia* species also exhibited good antimycobacterial activity with MIC values less than 1 mg/ml. The ethanol extract of *S. aethiopis* and compounds isolated from *S. multicaulis* (eg. multiorthoquinone) have been reported to display promising activity against *M. tuberculosis* (MIC values: 50 and 2 µg/ml, respectively) (Cantrell *et al*., 2001; Tosun *et al*., 2004). Seaman (2005) found that a *S. africana-lutea* extract exhibited promising activity against *M. aurum* and *M. tuberculosis* (MIC values: 2 and 1 mg/ml, respectively) along with moderate activity against *M. smegmatis* (MIC value: 8 mg/ml).

Salvia species are rich in phenolic compounds and the latter are known to display a broad range of biological activities such as antibacterial, anti-oxidant, anticancer and anti-inflammatory activities (Lu and Foo, 2001; Ulubelen *et al*., 2001; Yamazaki *et al*., 2004). The species investigated revealed high phenolic content (Chapter 5) may contribute to the observed activity.

The essential oils showed moderate antibacterial activity, especially against *B. cereus,* while little activity was obtained against *S. aureus* (Table 9.1). The essential oils of many Lamiaceae have been known since antiquity to display various biological activities, notably their antimicrobial potency (Barrata *et al*., 1998). The study of biological and pharmacological activities of essential oils in aromatherapy is highly correlated to the chemical composition (Lawrence, 2000). The results relating to the hydrocarbon and the

oxygenated fractions of *S. officinalis* essential oil showed that the hydrocarbon fractions did not inhibit the growth of micro-organisms, while the oxygenated fractions exhibited a significant dose response effect (Carta *et al*., 1996). Delaquis *et al*. (2002) found that alcohols and aldehydes were particularly active against Gram-positive bacteria. Purified compounds derived from essential oils, such as carvacrol, borneol, geraniol, eugenol, linalool, camphor, 1,8-cineole and thymol, inhibit a wide variety of micro-organisms, while α-pinene (monoterpene hydrocarbon) had slight activity against a range of micro-organisms (Hinou *et al*., 1989; Zakarya *et al*., 1993; Carta *et al*., 1996; Tzakou *et al*., 2001). The low potency (MIC values > 32 mg/ml) observed for some of the essential oils may possibly be due to the high concentration of hydrocarbon components (15-56%), while the moderate activity (MIC < 8 mg/ml) may be correlated to the oxygenated components present in the oils (22-74%) (Table 4.1). Compounds such as *p*-cymene, α-pinene and limonene have been previously been shown to exhibit low antibacterial activity (Chalchat *et al*., 1997). The relationship between composition and biological activity suggests that the compounds present in the greatest proportions (e.g. linalool, linalyl acetate, α-bisabolol, limonene, 1,8-cineole, α-pinene) are not necessarily responsible for the greatest share of the total antibacterial activity. Thus, the involvement of the less abundant constituents (e.g. β-caryophyllene alcohol, terpinen-4-ol) should be considered. Essential oils could possibly be rendered more useful if the relative concentrations of antimicrobial components were adjusted to levels that consistently provide the required potency and spectrum of inhibition (Delaquis *et al*., 2002), but the safety levels also need to be considered. Several factors may contribute to the disparity in observations with reported activity of essential oils. Variability in qualitative and quantitative estimates of activity has been ascribed to differences in analytical techniques (Mann and Markham, 1998). The volatility and poor solubility of most essential oils is problematic, particularly with methods that rely on dilution of the test substance in an aqueous medium (Delaquis *et al*., 2002). Furthermore, the essential oils are volatile and one has to take into account a loss of the active substances, especially when the incubation period is long (Pauli, 2001). They are complex mixtures comprising many individual compounds and each of these constituents may contribute to the beneficial or adverse effects of these oils (Lahlou, 2004).

It was interesting to note in the current study that the activity of the essential oil of *S. africana-caerulea* was higher than that of the solvent extract (Table 9.1). In many studies,

the antibacterial activity of solvent extracts is generally high compared to that of essential oils (Lourens *et al*., 2004; Njenga *et al*., 2005, van Vuuren *et al*., 2006). *Eriocephalus punctulatus* and *E. ericoides* essential oils were also found to exhibit better activity in comparison to the acetone extract (Njenga *et al*., 2005). Lourens *et al*. (2004) also observed that *Helichrysum excisum* essential oil was more active than the methanol and acetone extract.

The Gram-positive bacteria were found to be more sensitive than the Gram-negative bacteria (Table 9.1) which is in agreement with other studies (Delaquis *et al*., 2002; Viljoen *et al*., 2003; Njenga *et al*., 2005). Reasons for the resistance of many Gram-negative bacteria will likely remain speculative until the mode of action is better understood. Current hypotheses favour adverse effects on the integrity of the bacterial cell membrane (Delaquis *et al*., 2002). Studies have reported that the sensitivity of bacteria was related to the morphological structure and chemical composition of bacteria membranes. Gram-negative bacteria in general have an outer membrane composed mainly of polysaccharides which is rather impermeable, thereby preventing inhibitors from passing through (Nikaido and Vaara, 1985). Given the differences in the cell envelope of the Gram-negative bacteria, it is plausible that access through the cell membrane is more restricted in Gram-negative bacteria.

Infections caused by *S. aureus* are among the most difficult to treat with standard antibacterial agents (Tomás-Barberán *et al*., 1990). The growth of *S. aureus* was inhibited by nearly all the extracts (Table 9.1), especially by *S. aurita*, *S. chamelaeagnea*, *S. dolomitica*, *S. radula* and *S. stenophylla* (MIC values $\leq$ 0.06 mg/ml). Thus, these plants could yield compounds that can improve the treatment of infections caused by *S. aureus* in particular. It was reported that many species of *Salvia* are used in decoctions or infusions in South Africa by Sotho people against possible bacterial infections such as throat inflammation, colds, coughs and to treat pulmonary diseases and tuberculosis (Watt and Breyer-Brandwijk, 1962). The *in vitro* antibacterial and antimycobacterial activity obtained in this study strongly support the traditional medicinal uses of these plants.

As a rule, a single group of compounds dominates within a given taxon with major compounds often accompanied by several derivatives and minor components (Wink, 2003). The biological activity of any plant is primarily a function of the type of

compounds present. Those isolated from *S. chamelaeagnea* as well as some standards (rosmarinic acid, carnosic acid and caffeic acid) known to display antimicrobial activity were also present in most of the investigated *Salvia* species (Table 5.2) and have probably contributed to the observed activity. More precisely, some of the isolated compounds contain phenolic hydroxyl groups which are reported to be associated with antibacterial activity (Tomás-Barberán *et al*., 1990). The MIC values of the isolated compounds indicated promising activity against *S. aureus* and to a certain degree, against *B. cereus*. The solvent extracts are crude preparations and one should expect the activity of the isolated molecules to be higher than the crude extracts. This was not observed and may partly be attributed to the lability of the isolated compounds. Furthermore, it is not possible to quantify the antibacterial activity from bio-autographic assay where the inhibition spot is by nature a positive or negative response and does not reveal detail about the degree of activity of a specific compound. The mechanism of many phytomedicines is still unknown and there are several instances of total herb extract showing a better effect than an equivalent dose of an isolated compound.

The active isolated compounds have previously been detected in many species of the Lamiaceae. Carnosol has been reported to display antibacterial activity against various micro-organisms including *S. aureus*, *Bacillus subtillus*, *E. coli* and *Candida albicans* (Collins and Charles, 1987; Dimayuga *et al*., 1991). It is a common compound in *Salvia* species and was first isolated from *S. carnosa* (Dougl.) (White and Jenkins, 1942). Since then, this compound has been detected in *Rosmarinus officinalis* and many other species belonging to the Lamiaceae family (Wu *et al*., 1982).

A mixture of ursolic acid and its isomer, oleanolic acid, was previously isolated from *Swertia arisanensis* (Gentianaceae) (Lin *et al*., 1987) and the two compounds have been found in more than 120 plant species including the Lamiaceae family. Medicinal plants containing ursolic acid have been used in folk medicine before it was known which constituents were responsible for their therapeutic effectiveness (Liu, 1995). Contemporary scientific research that led to the isolation and identification of ursolic acid revealed and confirmed that several pharmacological properties such as antitumour, hepatoprotective, anti-inflammatory, anti-ulcer, antimicrobial, antihyperlipidemic and antiviral activity can be attributed to this compound (Liu, 1995). Numerous plants from the Lamiaceae family containing ursolic acid and its derivatives, exhibited antimicrobial

activity and these therapeutic effects have been confirmed by laboratory testing (Sattar *et al.,* 1995). Ursolic acid was also identified as one of the active principles in *Rosmarinus officinalis* that inhibited the growth of some food-borne pathogens (Collins and Charles, 1987). The isolated compounds have long been found in *Salvia* and other plants around the world. However, this is the first investigation reporting their presence in *S. chamelaeagnea*.

A major characteristic of traditional medicine that has remained constant is the use of plant mixtures to cure various diseases (Iwu, 1994). The mixtures of the active compounds in producing desired biological effects are common practice in health care and clinical treatment (Liu, 2005). Most of these phytomedicines are on the drug market as whole plants extracts. Synergistic interactions between the compounds of individual or concoctions of various plants are a vital part of the therapeutic efficacy of the traditional practitioners. The combination study involving *S. chamelaeagnea* and *L. leonurus* displayed synergistic or antagonistic interactions depending on the test organism (Table 9.2; Figure 9.5). Boik (2001) conducted a large number of combination studies using various natural substances and the results strongly suggest that when used in combination, natural substances can produce synergistic interactions *in vitro*. Phenolic compounds such as flavonoids may have a role to play in increasing the biological activity of other compounds by synergistic interactions (Williamson, 2001). The synergistic interactions obtained with the Gram-positive bacteria with nearly all ratios (Table 9.2; Figure 9.5) indicated that the combination of natural products might be an alternative approach in the development of new anti-infective drugs. However, one should note that antagonistic actions could also occur, as can be seen with the Gram-negative bacteria. Research on chemical mixtures has been difficult but it is important to fully understand the use of natural occurring compounds in the treatment of various diseases (Liu, 2005). The antimicrobial activity of plants used alone or in combinations may involve distinct active principles with distinctive mechanisms of action (Okoli and Iroegbu, 2004).

Although the investigations into the biological activities of chemical mixtures are difficult, it is important to fully understand the mechanisms by which these naturally occurring compounds are effective and interact in the treatment of many diseases (Liu, 2005).

9.5 Conclusions

- The solvent extracts and the essential oils displayed varying degrees of activity against bacteria, with the solvent extracts being more active than essential oils.
- *Salvia dolomitica*, *S. radula* and *S. verbenaca* exhibited the best activity against *M. tuberculosis,* while *S. chamelaeagnea*, *S. muirii*, *S. runcinata* and *S. stenophylla* displayed the most promising activity against the two Gram-positive and two Gram-negative bacteria.
- The synergistic interaction between *S. chamelaeagnea* and *L. leonurus* against *S. aureus* and *B. cereus*, indiates that the concurrent use of these two plants may broaden the therapeautic indications for *Salvia* species.
- Bioassay-guided fractionation led to the isolation of four compounds with antibacterial activity from *S. chamelaeagnea.*
- Carnosol, oleaonolic acid, ursolic acid and 7-*O*-methylepirosmanol inhibited the growth of micro-organisms to varying degrees.
- As carnosol, ursolic acid and oleanolic acid are known to exhibit good anti-oxidant activity, these compounds may prove a useful substitute for some of the phenolic anti-oxidants in current use, whilst offering an improved inhibitory capacity to microbial growth.

Chapter 10: *In Vitro* Anticancer Activity of the Solvent Extracts

Abstract

Salvia species are used in traditional medicine to treat various diseases including cancer. As part of an ongoing effort to verify the traditional uses of South African medicinal aromatic plants, 17 *Salvia* species were tested for *in vitro* anticancer activity. The experiment was conducted on three human cell lines, including the breast adenocarcinoma (MCF-7), the colon adenocarcinoma (HT-29) and the glioblastoma (SF-268) cell lines, using the sulforhodamine B assay. The results showed that the extracts inhibited cell proliferation of all three cell lines to varying degrees. The concentration required to inhibit 50% of cell growth (IC_{50} values) ranged between 9.69 and 43.65 µg/ml and between 8.72 and 59.12 µg/ml against the MCF-7 and SF-268 cell lines, respectively, with *S. radula* and *S. africana-caerulea* being the most active. IC_{50} values against the HT-29 cell line ranged from 17.05 to 57.00 µg/ml, with the extract from *S. lanceolata* being the most active. Cell line specificity was observed for *S. dolomitica* and *S. garipensis*, while *S. lanceolata*, *S. muirii*, *S. namaensis*, *S. repens*, *S. runcinata* and *S. verbenaca* showed some degree of cell-type selectivity. In the search for new anticancer agents, plants that display selective activity against specific cell lines should be further investigated.

10.1 Introduction

Cancer is caused by the uncontrolled growth and spread of cells that may affect almost any tissue of the body (WHO, 2006b). The ability of ionising radiation to provoke the development of cancer has long been recognized, and the direct absorption of energy by DNA, resulting in damage to this molecule, may be part of the basis for radiation-induced carcinogenesis. There is extensive, indirect evidence supporting the role of free radicals in the promotion of cancer, as many free radical-generating compounds are tumour promotors (Kehrer, 1993).

Cancer is a worldwide public health problem, with more than 11 million people being diagnosed every year. It is estimated that by 2020, there will be 16 million new cases annually. It is reported that cancer causes 7 million deaths each year and results in 12.5% of deaths worldwide (WHO, 2006b). Cancer produces no signs or symptoms that exclusively indicates the presence of the disease. Complaints such as haemorrhoids, anaemia, bronchitis, sinusitis, weight loss and fever may be associated with cancer. Some common symptoms of cancer include a persistent cough or blood-tinged saliva, a change in bowel movements, blood in the stool or urine, unexplained anaemia, breast lump or discharge, change in urination (frequent urination, a small amount of urine and/or weak urine flow), night sweats and unexpected weight loss (WHO, 2006b).

A major problem with the available cancer chemotherapy is the serious deficiency of active drugs for curative therapy and the resistance developed by many tumours to treatment with standard anticancer agents (Cragg and Newman, 2005) since the majority of cancer chemotherapeutic agents severely affect the 'host´s' normal cells (Mascarenhas, 1994). Plants have a long history of use in the treatment of cancer. In his review, Hartwell (1982) lists more than 3,000 plant species that have reportedly been used in the treatment of cancer, but in many instances the "cancer" is undefined or reference is made to conditions such as "hard swelling", abscesses, calluses, corns, to name a few (Cragg and Newman, 2005). Such symptoms would generally apply to skin or visible conditions, but many of the claims for efficacy should be viewed with some skepticism because cancer, as a specific disease entity, is likely to be poorly defined in terms of folklore and traditional medicine (Cragg and Newman, 2005; Steenkamp and Gouws, 2006). Despite these observations, plants have played an important role as a source of effective anticancer

agents and it is significant that over 60% of currently used anticancer agents are derived in one way or another from natural sources including plants, marine organisms and micro-organisms (Cragg *et al*., 1997; Valeriote *et al*., 2002). The first agents to advance into clinical use were the so-called vinca alkaloids, vinblastine and vincristine, isolated from the Madagascar periwinkle, *Catharanthus roseus* (Apocynaceae). Others compounds, such as paclitaxel (Taxol®), topotecan and irinotecan have been isolated. A number of promising new agents from plants including flavopiridol and combretastin are in clinical development based on selective activity against cancer-related molecular targets (Cragg and Newman, 2005). In South Africa, reports on plants used for the treatment of cancer are rare, and can be ascribed to the fact that cancer involves a complex set of signs and symptoms (Steenkamp and Gouws, 2006). With this in mind, it is recommended that when investigating plants for anticancer potential, ethnopharmacological properties such as immune and skin disorders and inflammatory diseases be taken into account when selecting plants used to treat cancer, since these could reflect disease states bearing relevance to cancer or a symptom thereof (Cordell *et al*., 1991; Popoca *et al*., 1998). Plants have been a prime source of highly effective conventional drugs for the treatment of many forms of cancer and, while the isolated compounds from the plants may not often serve as the drugs, they may provide leads for the development of potential novel agents (Cragg and Newman 2005). *Salvia* species have been used against various infectious and inflammatory diseases. Furthermore, they have been used in traditional medicines in China, South Africa and many other countries to treat symptoms associated with cancer (Watt and Breyer-Brandwijk, 1962; Ulubelen *et al*., 1999; Shoemaker *et al*., 2005).

There has been substantial progress in the development of anticancer drugs, but a number of common cancers appear to be resistant to the available drugs (e.g. Tamoxifen and Andriamycin™) (Salmon, 1984; Shoemaker *et al*., 2005). Furthermore, compounds were selected primarily on their gross cytotoxicity rather than on their selective ability to inhibit the growth of tumour cells (Valeriote *et al*., 2002). Another reason for the search for new anticancer drugs is the fact that current drugs have serious adverse effects.

The objectives of this study were to:

(i) evaluate the *in vitro* anticancer activity of the solvent extracts (Section 3.2.2) of *Salvia* species against three human cell lines using the sulforhodamine B assay,

(ii) assess any possible cell line specificity, and

(iii) support the use of indigenous *Salvia* species used in traditional medicine as anticancer agents.

10.2 Materials and methods

10.2.1 Chemicals and drugs

RPMI-1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), 0.25% trypsin-0.1% ethylene diamine tetra acetic acid (EDTA), fetal bovine serum (FBS), sodium pyruvate and 10 mg/ml penicillin G-sodium/streptomycin sulphate (100 x) were from Highveld Biological; sulforhodamine B (SRB), gentamicin sulfate and trypan blue were obtained from Sigma®. Trichloro-acetic acid (TCA) and DMSO were purchased from Saarchem. Tris (hydroxymethyl aminomethane) and the reference drug, 5´-fluorouracil, were purchased from Merck.

10.2.2 Preparation of media and cell maintenance

10.2.2.1 Preparation of different media and solutions

The routine culture medium used to maintain the SF-268 and MCF-7 cells was prepared using 470 ml of RPMI-1640, 25 ml of FBS and 5 ml of L-glutamine, while the experimental medium was prepared in the same manner with the addition of 500 µl of 0.1% gentamicin sulphate to prevent any contamination. The DMEM used to maintain the HT-29 cell line was prepared with 13.53 g DMEM and 3.7 g $NaHCO_3$ in one liter of distilled Millipore® water (further sterilised). Penicillin G-sodium/streptomycin sulphate (1 ml), sodium pyruvate (2 ml) and FBS (25 ml) were added to DMEM (470 ml) to constitute the routine culture medium.

The SRB solution was prepared at a concentration of 0.4% (w/v) in 1% acetic acid and the TCA solution made at a concentration of 50% (w/v) with distilled Millipore® water. Trypan blue was prepared at a concentration of 2 mg/ml in PBS (Section 8.2.2.2) and the solution of Tris (hydroxymethyl) aminomethane was prepared at a concentration of 10 mM (pH 10.5) with distilled Millipore® water. All solutions were stored at 4 ºC until required.

10.2.2.2 Source and maintenance of cell lines

Cells lines representing the most common human cancers (WHO, 2006b) were obtained from the National Cancer Institute (NCI). The cell lines include the breast adenocarcinoma (MCF-7), the glioblastoma (SF-268) and the colon adenocarcinoma (HT-29). They were chosen for the following reasons:

(i) adaptability to growth in RPMI-1640 or DMEM plus FBS with glutamine,
(ii) suitability for use in microculture, and
(iii) mass doubling time that allows for harvesting of approximately 1.5 x 10^5 cells/ml every week.

The three cell lines were cultured in 200 ml flasks (Nunclon™) with the complete culture medium (RPMI-1640 or DMEM). The cells were maintained at 37 ºC in a 5% CO_2 humidified incubator. The culture medium (20 ml) was replaced three times weekly and the cells were trypsinised when nearly confluent.

10.2.2.3 Preparation of cell suspension

All the cells were detached from the culture flasks by addition of 1 ml of 0.25% trypsin-0.1% EDTA. Trypsin was then inactivated by the addition of 10 ml of experimental medium (RPMI-1640 or DMEM). Cells were separated into a single cell suspension by a gentle pipetting action and 1 ml of the cell suspension was returned to the culture flask containing 20 ml of culture medium. The remaining cell suspension was centrifuged at 1000 rpm (Sorvall® T6000D) for 3 minutes and the supernatant discarded. Experimental medium (20 ml) was added to the cells which were then repeatedly pipetted to allow cells to disperse in to the medium. An aliquot of the trypsinised cell suspension was stained with 2 mg/ml trypan blue (1:1 ratio) in order to obtain a more quantitative analysis of the cell suspension. Trypan blue is a water-soluble dye, which is insoluble in the cell membrane lipids. It will thus only cross cell membranes of dead/non-viable cells. On visualisation with the light microscope (Nikon), viable cells appear transparent, while non-viable cells are stained dark blue. The haemocytometer was used to determine the total number of viable and non-viable cells by counting cells in the 25 squares (each square is subdivided into 16 smaller squares of 0.1 mm^2) at the top and bottom of the haemocytometer and the average number of cells per unit volume (millimeter, ml) of medium calculated. The cell suspension was adjusted with experimental medium to

approximately 1.5 x 10^5 cells/ml. The experiment was carried out only when there was at least 95% cell viability.

10.2.2.4 Preparation of test samples and standard

The stock solution (10 mg/ml) of each test sample (solvent extract and standard) was prepared with DMSO and a further 1:50 dilution was made with appropriate experimental medium. The final DMSO concentration in the well of 1% had no effect on the growth of the cells. Stock samples were not filtered because DMSO has a sterilizing effect. Stock solutions were stored at -20 ºC to provide consistency between tests. The essential oils were not tested against cancer cell lines due to the small amount of the oils available.

10.2.3 The sulforhodamine B assay

The colourimetric SRB microculture assay was performed according to Monks *et al*. (1991) and Wu *et al*. (1993). A volume of 100 µl cell suspension containing 150,000 cells/ml was seeded into a 96-well microtitre plate (final count 15,000 cells/well), except the blank and the test sample background colour (only sample plus experimental medium) wells where 100 µl of appropriate experimental medium was plated (Figure 10.1, rows A and H).

The effect of each concentration of the solvent extract on the cell line was performed in triplicate wells. For instance, sample 1 corresponds to wells B3-D3, while well A3 was used as its corresponding background colour control which contained test sample and experiemental medium (no cells) (Figure 10.1). Wells, which contained cells with experimental medium in the absence of the test samples (Figure 10.1, B1-G1; B2-G2), were used as drug-free controls, while blank wells (A1, A2, H1 and H2) contained experimental medium only.

The plate was incubated for 24 hours at 37 ºC, 5% CO_2 and 100% relative humidity to allow for cell attachment prior to the addition of the test samples. Thereafter, 100 µl of the different sample dilutions were added to the appropriate microtitre wells, resulting in the required final sample concentration. The plate was incubated for a further 48 hours. At the time of sample addition, a separate reference plate for cell growth at time zero (the time at which drugs were added) was also prepared as described above.

For each dilution, a control was also plated out (in order to take into consideration the absorbance resulting from the colour of extract with experimental medium alone: rows A and H (Figure 10.1). The final concentration in the wells ranged from 0.34 to 100 µg/ml for 5´-fluorouracil (5´-FU) and from 6.125 to 100 µg/ml for the solvent extracts.

At the end of the 48 hour incubation period, cells were fixed *in situ* by layering 50 µl of ice-cold TCA (50%, w/v) onto the medium in each well and incubating the plate for one hour at 4 ºC. The supernatant was then discarded and the plate was rinsed at least five times with tap water to remove the TCA, growth medium and low-molecular weight metabolites, before air drying the plate at room temperature.

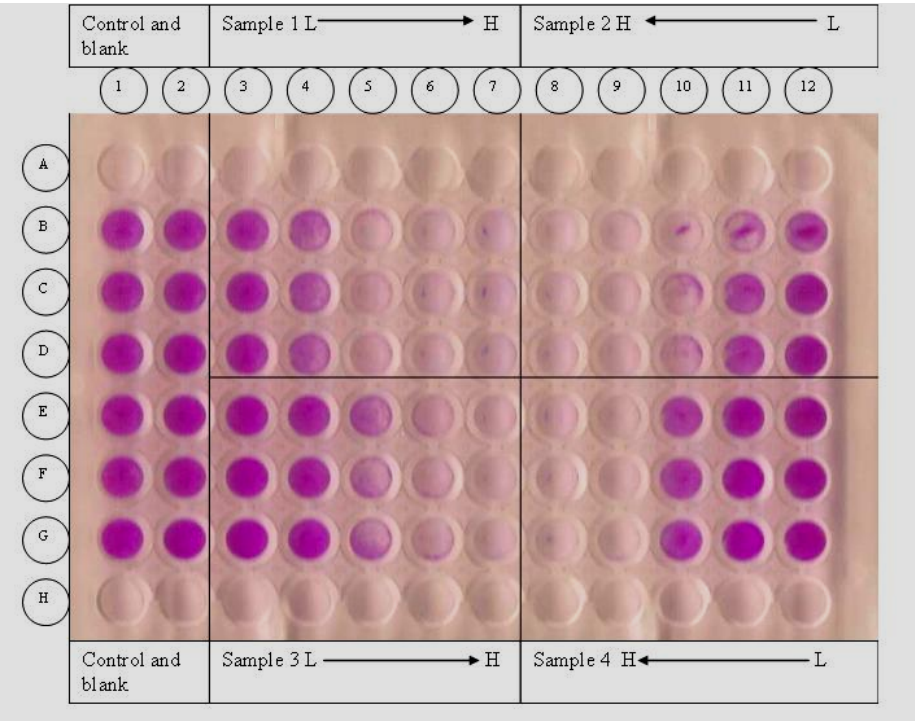


Figure 10.1 Microtitre plate showing how extracts were plated out in the anticancer assay and colour change of the SRB dye as a result of cell death. Each sample had five dilutions plated out in triplicate, plus one control well for measuring the colour of the sample in the presence of the experimental medium. The arrow indicates the direction of increasing concentration of the extract: L: low and H: high.

SRB is a water-soluble dye that binds to basic amino acids of cellular proteins that are only synthesised by viable cells (Voigt, 2005). The colour intensity in each well thus corresponds to the number of viable cells and inhibitory effect of the test compounds

(Figure 10.1). TCA-fixed cells were stained with 100 µl of SRB solution (0.4%, w/v). After 10-20 minutes at room temperature, the excess SRB solution was discarded and the cells washed thoroughly with 1% acetic acid to remove unbound dye. The plate was then air-dried at room temperature for approximately 2 hours until no moisture was visible. Bound dye was solubilised with 200 µl of Tris (hydroxymethyl) aminomethane (10 mM). The SRB solution has a maximum absorbance at 492 nm. The absorbance was read against a Tris (hydroxymethyl) aminomethane blank on an automated spectrophotometer plate reader (Labsystems iEMS reader MF) connected to the Ascent® version 2.4 software, at a single wavelength after the plate was shaken at 960 rpm for 3 minutes.

10.3 Data analysis

The percentage cell viability (in reference to control growth) to controls of the various extracts was calculated using the Microsoft Excel® programme (Eq. 10.1). The IC_{50} values (concentration at which 50% of cells were killed) were calculated from the log sigmoid-dose response curve using Enzfitter® version 1.05 software. The IC_{50} values were determined only for those extracts that displayed at least 80% inhibition when tested at 100 µg/ml. At least three independent runs were performed for each sample. One-way analysis of variance (ANOVA) and Student t-tests were used to compare data using Statistica version 5.0 software at a 95% confidence limit.

Percentage cell viability was calculated as follows:

$$\% \text{ Cell viability} = \frac{\text{Abs test sample} - \text{Abs background sample} - \text{Mean Abs blank}}{\text{Mean Abs control} - \text{Mean Abs blank}} \times 100 \quad \text{(Eq. 10.1)}$$

Where: Abs: absorbance at 492 nm

10.4 Results

The plant extracts exhibited anticancer activity against the HT-29, MCF-7 and SF-268 cell lines with the inhibitory effect increased as the concentration of the solvent extract increased (Figure 10.2). This pattern was observed for all solvent extracts against all the cell lines tested.

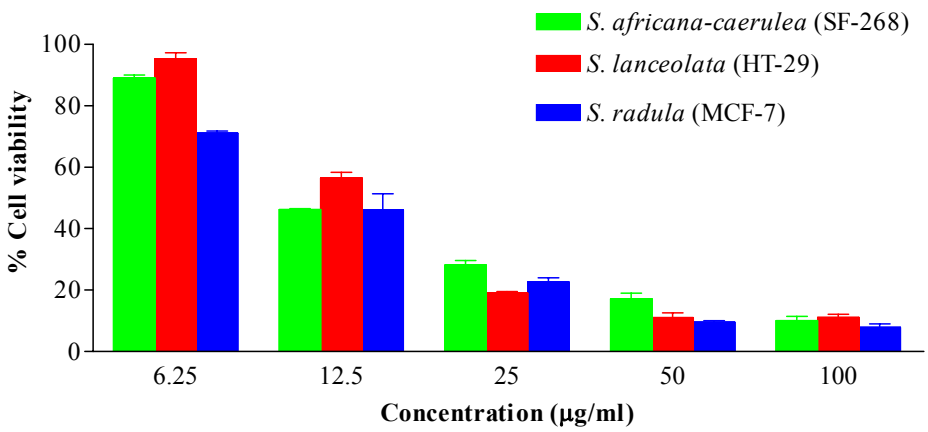


Figure 10.2 Percentage cell viability of cancer cells tested at various concentrations for three *Salvia* species (mean ± s.d., *n* = 3): *S. africana-caerulea* (SF-268), *S. lanceolata* (HT-29) and *S. radula* (MCF-7).

The inhibition of tumour cell proliferation by the solvent extracts of the 17 indigenous *Salvia* species investigated are displayed in Table 10.1. All the investigated solvent extracts were significantly less potent than 5′-FU ($P < 0.05$).

The best activity against the HT-29 cell line was obtained with the extracts of *S. lanceolata* and *S. stenophylla* (IC_{50} value: 17.05 ± 3.50 µg/ml) and *S. africana-lutea* (IC_{50} value: 20.00 ± 3.41 µg/ml), *S. aurita* (IC_{50} value: 24.58 ±6.41 µg/ml) and *S. namaensis* (IC_{50} value: 24.39 ± 3.42 µg/ml) which showed no significant difference in their activity ($P > 0.05$). Five species, viz *S. africana-caerulea*, *S. albicaulis*, *S. aurita*, *S. chamelaeagnea*, *S. disermas* and *S. namaensis*, did not differ significantly from one another in the inhibitory effects ($P > 0.05$).

S. radula exhibited the most favourable activity (IC_{50} value: 9.69 ± 0.92 µg/ml) ($P < 0.05$), while *S. africana-lutea* and *S. runcinata* were amongst the least active against the MCF-7 cells (Table 10.1). ANOVA analysis showed no significant difference in the activity exhibited by *S. aurita*, *S. chamelaeagnea*, *S. schlechteri* and *S. verbenaca* against the MCF-7 cells ($P > 0.05$). Similarly, the activity against the MCF-7 cell line did not differ for the following species: *S. africana-lutea*, *S. albicaulis*, *S. disermas*, *S. dolomitica*, *S. garipensis*, *S. muirii, S. namaensis*, *S. runcinata* and *S. verbenaca* ($P > 0.05$). On average, all of the 17 species showed an IC_{50} value of less than 44 µg/ml for the MCF-7 cells, divided as follows: four species, viz. *S. aurita, S. chamelaeagnea*, *S. radula* and *S.*

schlechteri with IC_{50} values less than 20 µg/ml; four species, viz *S. africana-caerulea, S. lanceolata, S. repens* and *S. stenophylla* had IC_{50} values ranging from 20 to 30 µg/ml, while the IC_{50} values of the remaining species were greater than 30 µg/ml. Two species, *S. dolomitica* and *S. garipensis,* also showed a degree of cell selectivity, as they were not active against the HT-29 (IC_{50} value > 100 µg/ml) and SF-268 (IC_{50} value > 100 µg/ml), but active against the MCF-7 cell line (IC_{50} value: 39 ± 4 µg/ml). None of the extracts were as active as 5′-FU ($P < 0.05$).

Table 10.1 The anticancer activity (IC_{50} values) of the solvent extracts of *Salvia* species and reference compound obtained against three human cancer cell lines.

Species / Reference	HT-29	MCF-7	SF-268
	IC_{50} values in µg/ml		
S. africana-caerulea	27.10 ± 1.08	23.36 ± 4.20	8.72 ± 1.52
S. africana-lutea	20.00 ± 3.41	43.65 ± 8.38	nc
S. albicaulis	25.33 ± 1.11	35.25 ± 5.66	27.50 ± 0.89
S. aurita	24.58 ± 6.41	17.28 ± 1.71	44.87 ± 2.48
S. chamelaeagnea	29.53 ± 5.14	18.12 ± 1.67	34.98 ± 4.07
S. disermas	26.87 ± 0.57	38.56 ± 1.28	59.12 ± 2.75
S. dolomitica	nc	37.05 ± 2.10	nc
S. garipensis	nc	39.44 ± 3.90	nc
S. lanceolata	17.05 ± 3.50	26.15 ± 2.15	nc
S. muirii	55.63 ± 1.30	39.07 ± 2.86	nc
S. namaensis	24.39 ± 3.42	36.36 ± 3.07	nc
S. radula	32.10 ± 2.93	9.69 ± 0.92	27.55 ± 4.52
S. repens	43.62 ± 5.17	23.36 ± 2.16	nc
S. runcinata	55.37 ± 2.10	43.42 ± 5.46	nc
S. schlechteri	57.00 ± 11.67	18.37 ± 0.47	54.40 ± 4.42
S. stenophylla	17.41 ± 2.65	23.74 ± 1.96	43.86 ± 3.43
S. verbenaca	50.04 ± 5.39	31.50 ±13.70	nc
5′-FU	7.00 ± 2.02	1.11 ± 0.31	nc

nc: IC_{50} values not calculated because the percentage inhibition at 100 µg/ml was less than 80%

In contrast, *S. africana-caerulea*, with an IC_{50} value of 8.72 ± 1.52 µg/ml, was the most active extract against the SF-268 cells ($P < 0.05$). The inhibition by *S. africana-lutea*, *S. dolomitica*, *S. garipensis*, *S. lanceolata*, *S. muirii*, *S. namaensis*, *S. repens, S. runcinata* and *S. verbenaca* tested at a concentration of 100 µg/ml exhibited less than 80% inhibition of the cell growth. The IC_{50} values were thus not calculated for these species (Table 10.1). The IC_{50} values of nearly all the species were above 30 µg/ml. No significant difference in the activity of *S. albicaulis* and *S. radula* against the SF-268 cells ($P > 0.05$) was observed. 5´-Fluorouracil was not active againt against the SF-268 cell line, while *S. africana-caerulea*, *S. albicaulis* and *S. radula* exhibited some degree of activity (IC_{50} value < 30 µg/ml) (Table 10.1).

Treatments with *Salvia* extracts resulted in growth inhibition in the majority of cases. For the three cell lines examined, MCF-7 cells were the most sensitive, as they wewre inhibited by all the solvent extracts, while the SF-268 cell line was the least sensitive. About 53% (9/17) of samples exhibited less than 80% inhibition at 100 µg/ml (Table 10.1).

In the screening of the anticancer activity of plants, species which need to be considered for further analysis are those that show activity against certain cell lines and are not toxic to others. In this study, cell line selectivity was observed. The solvent extracts of *S. dolomitica*, *S. garipensis*, *S. lanceolata*, *S. muirii*, *S. namaensis*, *S. repens*, *S. runcinata* and *S. verbenaca* showed some degree of activity against MCF-7, but were, however, not active against the SF-268 and/or HT-29 cell lines (Table 10.1).

Ursolic acid and oleanolic acid were isolated from *S. chamelaeagnea* as an inseparable mixture (Chapter 9). Ursolic acid (standard) exhibited low activity (IC_{50} value > 100 µg/ml), while its isomer, oleanolic acid (standard), displayed moderate activity against the MCF-7 cells (IC_{50} value > 72.77 ± 5.23 µg/ml). Carnosol, also isolated from *S. chamelaeagnea*, inhibited HT-29 cells with an IC_{50} value of 11.79 ± 0.94 µg/ml. This activity was, however lower than that of 5´-FU (IC_{50} value: 7.00 ± 2.02 µg/ml). Salvigenin, a compound isolated from *S. radula* (Chapter 8), exhibited moderate activity against the MCF-7 cells (IC_{50} value: 67.78 ± 3.78µg/ml).

10.5 Discussion

Some phytochemicals such as flavonoids and alkaloids, present in medicinal plants, are reported to possess substantial anti-oxidant, antibacterial, anti-inflammatory and anticarcinogenic activities. These phytochemicals have been explored extensively for their potential in the treatment of cancer. Well-known compounds like paclitaxel, a diterpenoid from *Taxus brevifolia*, and vincristine, an alkaloid from *Catharanthus roseus*, are used in chemotherapy today (Wall and Wani, 1996). Many *Salvia* species have been used in traditional medicine in South Africa, in Chinese herbal medicine, as well as in many other countries for the treatment of cancer (Watt and Breyer-Brandwijk, 1962; Ryu *et al*., 1997). The *in vitro* anticancer activity of indigenous South African species has, however, not yet been fully investigated.

The organic extracts of indigenous *Salvia* species were shown to have inhibitory effects on cancer cells in an apparent dose-dependent manner (Figure 10.2). In this study, there may be compounds in *Salvia* extracts that may induce a cytotoxic action against various cancer cell lines and initiate cell death. The American National Cancer Institute guidelines set the limit of activity for crude extracts at a 50% inhibition (IC_{50} value) of proliferation of less than 30 µg/ml (Suffiness and Pezzuto, 1990). The extracts exhibited various responses depending on the cell line used. The percentage of plant extracts with IC_{50} values less than 30 µg/ml was 17%, 47% and 53% against the SF-268, MCF-7 and HT-29 cells, respectively. *S. africana-caerulea* was the only species to exhibit anticancer activity against all three cell lines with an IC_{50} value of less than 30 µg/ml (Table 10.1).

The extract of *S. hypargeia* has been investigated by Ulubelen *et al*. (1999) against a panel of cell lines, including the human colon cells (COL-2), breast cancer cells (BC-1) and lung cancer cells (LU-1). Results indicated that the plant extracts possess anticancer activity (IC_{50} values < 20 µg/ml), which is lower than values obtained in this study, when tested against the breast cancer cell line. Other members of the family Lamiaceae have also been shown to inhibit the *in vitro* or *in vivo* growth of cancer cells. Ye *et al*. (2002) found that *Scutellaria baicalensis* (Lamiaceae) inhibited the growth of the most common human cancers, including breast cancer (MCF-7), colon cancer (HCT-15) and hepato-cellular carcinoma (HepG-2), with IC_{50} values of 1.1, 0.9 and 1.5 mg/ml, respectively, when tested *in vivo* in rats. Like most members of the Lamiaceae family, *Salvia* species are aromatic in

nature and are rich in essential oils (Chapter 4). Foray *et al*. (1999) investigated the *in vitro* anticancer activity of essential oils of *S. lavandulifolia*, *S. officinalis* and *S. sclarea* against various cancer cell lines and recorded IC_{50} values ranged from 0.6×10^{-4} µg/ml to 4.4×10^{-1} µg/ml. This higher cytotoxicity of the essential oil is due to the fact that several essential oil compounds have highly hydrophobic character. Their accumulation in the cellular membrane perturbs the membrane, resulting in increased permeability to protons and ions and eventually cell-death (Sikkema *et al*., 1995).

Compounds with anticancer properties have been isolated from many *Salvia* species and include cryptotanshinone, methylenetanshiquinone and tanshindiol A from *S. miltiorrhiza* (Ryu *et al*., 1997). These compounds were tested against the non-small cell lung (A549) and central nervous system cell lines. The IC_{50} values obtained less than 0.7 µg/ml, but were still less potent than the reference compound used in the study (doxorubicin, IC_{50} value < 0.1 µg/ml).

Some of the plants investigated in this study have been studied from a chemical point of view. Phytochemical research revealed that the principal secondary metabolites of *Salvia* species are mainly found to be flavonoids which are responsible for the antibacterial, anti-oxidant, anti-inflammatory, antimalarial and anticancer activities (Ulubelen *et al*., 1999; Kobayashi *et al*., 2002). Terpenoids have been isolated from many *Salvia* species, including this study (Chapter 9) and this class of compounds probably contributes to the anticancer activity exhibited by the extracts. Ursolic acid and oleanolic acid isolated from *S. chamelaeagnea* are shown to have marked anti-tumour effects, with the activity of ursolic acid being greater than that of oleanolic acid against the human colon carcinoma cell line LCT-15 (Li *et al*., 2002). *S. chamelaeagnea* was not, however, the most active extract. In this study, it was found that ursolic acid (standard) exhibited low activity (IC_{50} value > 100 µg/ml), while its isomer, oleanolic acid, displayed moderate activity (IC_{50} value: 72.77 ± 5.23 µg/ml) against the MCF-7 cell line. This is in agreement with the study conducted by Cragg and Newman (2005), which showed that ursolic and oleanolic acids are associated with weak anti-inflammatory and antitumour activities. Carnosol, also isolated from *S. chamelaeagnea* (Chapter 9), inhibited the HT-29 colon adenocarcinoma cells with an IC_{50} value of 11.79 ± 0.97 µg/ml. All the isolated compounds tested in this study were significantly less active ($P < 0.05$) than 5´-FU. Oleanolic acid, ursolic acid acid

and carnosol are good anti-oxidant (Chapter 5, Cuvelier *et al*., 1994) and may also offer an improved inhibitory capacity to cancer cells.

Flavonoids are biosynthesised by plants and have strong anti-oxidant activity for scavenging free radicals which are involved in cell damage and tumour promotion. Epidemiological studies suggest that flavonoids, such as the isoflavone genistein, play an important role in the prevention of carcinogenesis (Barnes, 1995). Indigenous *Salvia* species also possess flavonoids (Chapter 5) and one flavonoid has been isolated from *S. radula* as part of this research (Chapter 8). The presence of flavonoids may also contribute to the anticancer activity. The flavonoid isolated from *S. radula* (salvigenin) was tested against the MCF-7 cells and it exhibited moderate activity, although not significantly comparable to the activity of 5′-FU (IC_{50} value: 67.78 ± 3.78 µg/ml). Testing could not be done against the HT-29 and SF-268 cells due to the small amount of flavonoid isolated.

This is the first *in vitro* investigation to determine the anticancer activity on indigenous *Salvia* species. The investigated species have shown their ability to inhibit the proliferation of three human cancer cells. Furthermore, the selectivity of some of the solvent extracts indicated that *Salvia* species could be considered in the search for novel anticancer lead compounds.

10.6 Conclusions

- *Salvia* species inhibited the growth of three different cancer cell lines in a dependent manner.
- The best activity against the HT-29 cells was obtained with extracts obtained from *S. africana-lutea*, *S. aurita*, *S. lanceolata*, *S. namaensis* and *S. stenophylla*.
- The best activity against the MCF-7 and SF-268 was obtained with extracts from *S. radula* and *S. africana-caerulea*, respectively.
- Solvent extracts of *S. africana-lutea*, *S. dolomitica*, *S. garipensis*, *S. lanceolata*, *S. muirii*, *S. namaensis*, *S. repens*, *S. runcinata* and *S. verbenaca* were active against the MCF-7 cell line, but not active against the SF-268 and/or the HT-29 cell line.
- None of the solvent extracts were as effective as 5′-fluorouracil against MCF-7 and HT-29. The 5′-fluorouracil did not inhibit SF-268 cells, but *S. africana-caerulea*, *S. albicaulis* and *S. radula* inhibited it with an IC_{50} value of less than 30 µg/ml

(NCI cut off) and other species were more active against the cell line that is resistant to the effects of 5´-fluorouracil.

- The MCF-7 cell line was the most sensitive cell line, with 80% of cell growth being inhibited by a concentration of 100 µg/ml of the solvent extracts.
- The SF-268 cell line was the least sensitive cell line as it was inhibited by only 47% of the extracts when tested at 100 µg/ml.
- This study intends to further investigate *Salvia* species in the search for anticancer compounds and could help to explain their use in traditional medicine as chemotherapeutic or anticancer agents.

Chapter 11: *In Vitro* Evaluation of Toxic Effects of the Essential Oils and Solvent Extracts

Abstract

The *in vitro* toxicity profile of 28 samples (17 solvent extracts and 11 essential oils) was evaluated on human kidney epithelial cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5 dimethyl tetrazolium bromide (MTT) colourimetric method. The samples displayed some degree of toxicity with IC_{50} values ranging from 1.79 ± 0.43 to 22.9 ± 5.28 µg/ml for the essential oils and from 12.12 ± 2.02 to 53.34 ± 3.90 µg/ml for the solvent extracts. The toxicity profile of the essential oils was significantly higher compared to the solvent extracts ($P < 0.05$). The essential oil of *S. runcinata* (IC_{50} value: 1.79 ± 0.43 µg/ml) and the solvent extract of *S. stenophylla* (IC_{50} value: 12.12 ± 2.02 µg/ml) were the most toxic, while the *S. disermas* extract (IC_{50} value: 53.34 ± 3.90 µg/ml) and *S. radula* essential oil (IC_{50} value: 22.90 ± 5.28 µg/ml) were least toxic. The essential oils and solvent extracts were significantly more toxic than 5´-fluorouracil ($P < 0.05$).

11.1 Introduction

In recent years, there has been a worldwide trend towards the use of natural products as opposed to synthetic ones for medicinal purposes. Research and identification of active constituents are important in the search for new biologically active compounds in the pharmaceutical field. Many species of the Lamiaceae have potential therapeutic activities, which include anti-oxidant, anti-inflammatory, anticholinesterase, antibacterial and antimalarial activities (Cuvelier *et al.,* 1996; Ulubelen *et al*., 2001; Hayes and Markovic, 2002; Perry *et al*., 2003; Benoit-Vical *et al*., 2003). *Salvia* species are also used extensively in perfumery and aromatherapy (Baylac and Racine, 2003; Kaya *et al*., 2003). The common notion among people who use *Salvia* to treat various conditions is that ' if it does not benefit, it would not harm' (Gali-Muhtasib *et al*., 2000) and many people are not aware that excessive intake of sage extracts can be toxic and can lead to convulsions and permanent brain damage (Arnol, 1988). The increased worldwide use of plants in aromatherapy and pharmaceutical fields has raised a number of concerns in relation to their adverse effects, which warrants further investigation (Woolf, 1999; Thompson and Wilkinson, 2000). Poisoning by traditional remedies, especially by plants is well documented, particularly in South Africa (Joubert, 1990). But only a few plants used in traditional medicine contain pharmaco-active chemicals which are toxic. However, these make a significant contribution to morbidity and mortality (Steenkamp, 2000). In order to verify whether the biological activities (especially the anticancer activity) exhibited by plants are not correlated to their general cytotoxicity, the evaluation of their toxic effects against a normal cell line is necessary.

Plant material contains a large number of detectable and potentially active compounds, but only a few are responsible for the major pharmacological or adverse effects. Such compounds may be expressed in the plant at different levels depending on the location, soil in which it is growing and the season (Steenkamp, 2000). In addition, the toxic principle(s) may be located in only one part of the plant (e.g. leaves, seeds, roots or bark). Toxic compounds of plants are divided into various groups according to their chemical properties (e.g. alkaloids and glycosides) with some *Salvia* species having tested positive for alkaloids (Raffauf, 1996). Several studies on biological activities of *Salvia* species have been published (Cuvelier *et al*., 1994; Ulubelen *et al*., 2001; Baricevic *et al*., 2001; Tepe *et al*., 2006), but only a few have been conducted on the toxicity of these plant

species and may therefore place limitations on their use in conventional medicine. In phytomedicine as well as in clinical medicine, the toxicity profile of a given substance must always be evaluated, although plants found to be toxic *in vitro* may not necessarily be toxic *in vivo* because of the complexity of the human body. The lack of information on the toxic profile of *Salvia* species and the pharmacological activities exhibited in previous Chapters prompted this investigation in order to determine whether they are safe for use as unconventional medicines.

The objectives of this chapter were to:

(i) evaluate the toxicity profile of the essential oils and the solvent extracts using the MTT assay, and

(ii) verify the safety of *Salvia* species in traditional medicine.

11.2 Materials and methods

11.2.1 Chemicals, reagents and reference compound

Trypsin containing EDTA, fetal calf serum (FCS) and Ham F10 were obtained from Highveld Biological, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from USB®. $NaHCO_3$, gentamicin sulfate, trypan blue and 5′-fluorouracil were obtained from Sigma®.

11.2.2 Preparation of various media and solutions

11.2.2.1 Culture medium and experimental medium

The Ham F10 solution consisted of 9.38 g of Ham F10 medium and 1.18 g of $NaHCO_3$ in a total volume of 1 liter of Millipore® water and the solution was then filtered through a Sterrivex-GS 0.22 μm filter unit. The culture medium consisted of Ham F10 solution, gentamicin sulfate (10 mg/ml in PBS) and FCS in a proportion of 96:1:5, respectively. The experimental medium was prepared in the same way as the culture medium, except that gentamicin sulfate was not included.

11.2.2.2 Preparation of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide solution

The MTT solution was prepared at a concentration of 50 mg/ml with the PBS (Section 8.2.2.2), filtered and stored at 4 °C until used.

11.2.3 Cell maintenance

Human kidney epithelial cells were maintained continuously in a 500 ml flask (Nunclon™) with 50 ml of culture medium. The medium was changed thrice weekly or when it turned yellow. The cells were maintained at 37 °C in a 5% CO_2 humidified incubator and were trypsinised when nearly confluent.

11.2.4 Cell suspension and sample preparations

11.2.4.1 Single cell suspension

The cells were detached from the flask using 4 ml of trypsin (0.25%) containing EDTA (0.1%) after which 6 ml of the experimental medium was added to neutralize the effects of the trysin. The solution was gently agitated with a pipette until all the cells were dispersed into a single cell suspension. Subsequently, 1 ml of the cell suspension was returned to the culture flask with 49 ml culture medium. The remaining cell suspension was centrifuged at 1500 rpm for 5 minutes (Sorvall® T6000D), the supernatant-trypsin was discarded and 20 ml experimental medium added to the cells which were again agitated with a pipette until a single cell suspension was obtained. The cell density was determined using the trypan blue exclusion assay (Section 10.2.2.3), with 20 µl of cell suspension being stained with an equal volume trypan blue. The cells were counted using the haemocytometer and adjusted with the experimental medium to prepare a working solution of approximately 0.25 million cells/ml.

11.2.4.2 Preparation of samples

The stock solution of the solvent extracts and the 5′-FU were prepared with DMSO at a concentration of 10 mg/ml and stored at -20 ºC. The dilutions were prepared with experimental medium on the day of the experiment and the concentrations of the dilutions used were dependent on the results obtained from the initial broad screening. The toxicity profile of the isolated compounds from *S. radula* (salvigenin and betulafolientriol oxide)

and *S. chamelaeagnea* (carnosol); as well as the commercial standards of ursolic and oleanolic acid were also tested. These samples were prepared in the same as the solvent extracts.

The stock solution of each essential oil was prepared at a concentration of 10 mg/ml with DMSO and the dilutions were also prepared with DMSO on the day of the experiment. The final concentration of DMSO (1%) in the wells had no effect on the normal growth of cells. Seven dilutions were considered for each sample. The final concentration in the wells ranged from 1 to 100 µg/ml for the solvent extracts and 1 to 50 µg/ml for the essential oils. Concentrations of the reference compound varied from 1 to 500 µg/ml. Because essential oils are volatiles, they were always tested on separate microtitre plates and placed in different incubators to the solvent extracts.

11.2.4.3 The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay

The MTT colourimetric assay was performed on human kidney epithelial cells according to the method of Mosmann (1983) taking into consideration modifications by van Zyl and Viljoen (2002).

For this assay, a volume of 180 µl of the cell suspension was plated out into a 96-well plate (Nunclon™), except for two wells which served as the blank controls to which 180 µl of experimental medium were added. In order to test the effect of the solvent on the cells, two wells containing 1% DMSO were also included. After six hours of incubation (to allow for cell attachment), 20 µl for each dilution for the solvent extracts and reference compound or 2 µl of the essential oils, were added in triplicate to a 96-well culture plate. The experimental medium (18 µl) was added to the wells containing essential oils to ensure a total volume of 20 µl in each well. In the drug-free control wells (those only containing 180 µl of cells), 20 µl of experimental medium was added. The plate was then incubated in a humidified environment at 37 ºC and 5% CO_2 for 44 hours.

The validity of the MTT assay depends on the assumption that only viable cells reduce tetrazolium salts to coloured formazan which can be quantified spectrophotometrically (Mosmann, 1983). After 44 hours of incubation, 40 µl of the MTT solution (50 mg/ml) was added to each well and the plate incubated for a further four hours. Thereafter, 200 µl of the contents of each well was removed and discarded. Subsequently, 150 µl of DMSO

was added to each well to stop the reaction and dissolve the formazan crystals. The plate was then shaken at 1020 rpm for four minutes and the absorbance was read on an automated spectrophotometer plate reader (Labsystems iEMS reader MF) connected to the Ascent® version 2.4 software at the test wavelength of 540 nm and the reference wavelength of 690 nm.

11.3 Data analysis

The percentage cell viability was determined using a simple programme written in Microsoft Excel® by comparing the absorbance between the test samples and the controls (Eq. 11.1).

$$\% \text{ Cell viability} = \frac{\text{Abs of test sample} - (\text{Mean Abs of control} - \text{Mean Abs of blank}) \times 100}{\text{Mean Abs of controls} - \text{Mean Abs of blank}} \quad \text{(Eq. 11.1)}$$

Where: Abs = absorbance

Abs of test sample = Abs_{540} of test sample – Abs_{690} of test sample

Abs of control = Abs_{540} control – Abs_{690} control

Abs of blank = Abs_{540} of blank – Abs_{690} of blank

The IC_{50} values (concentration at which 50% of cell viability is obtained) were determined using Enzfitter® version 1.05 software. At least three independent runs were performed for each sample and results are given as mean ± s.d. An ANOVA and Student t-tests were used to analyse results. A correlation between the anticancer activity and toxicity profile was assessed using Pearson's correlation coefficient. All statistical analyses were performed using Statistica® version 5.0 software at 95% confidence limits. The toxicity of each sample was compared to that of the 5′-FU (ratio of toxicity of the 5′-FU to toxicity of the test sample). For the toxicity assay, the lower the IC_{50} values, the more toxic the sample.

11.4 Results

The toxicity profile of the essential oils, solvent extracts and the reference compound are depicted in Table 11.1. The essential oils and solvent extracts showed diverse levels of toxicity. The IC_{50} values ranged from 1.79 ± 0.43 to 22.90 ± 5.28 μg/ml for the essential oils. Of all the plants screened, the toxicity profile of the essential oil of *S. africana-*

caerulea, *S. runcinata* and *S. stenophylla* were similar ($P > 0.05$) and were also the most toxic ($P < 0.05$).

Table 11.1 The toxicity profile of the essential oils, solvent extracts and reference compound.

Species/Reference compound	**Essential oils** (IC_{50} values in µg/ml)	**Solvent extract** (IC_{50} values in µg /ml)	**Safety Index**	
			SI_1	**SI_2**
S. africana-caerulea	1.87 ± 0.52	14.38 ± 3.39	72.75	9.46
S. africana-lutea	7.24 ± 0.67	25.01 ± 1.94	18.79	5.44
S. albicaulis	2.75 ± 0.72	37.29 ± 0.58	49.47	3.64
S. aurita	nd	28.31 ± 4.94	nd	4.80
S. chamelaeagnea	6.00 ± 0.74	24.76 ± 7.60	22.67	5.49
S. disermas	nd	53.34 ± 3.90	nd	2.55
S. dolomitica	7.74 ± 0.27	40.26 ± 9.07	17.57	3.37
S. garipensis	nd	42.44 ± 3.03	nd	3.20
S. lanceolata	3.66 ± 0.92	26.71 ± 6.41	37.17	5.09
S. muirii	3.09 ± 0.53	37.00 ± 5.41	44.03	3.68
S. namaensis	nd	21.91 ± 2.89	nd	6.21
*S. radula**	22.90 ± 5.28	20.12 ± 4.02	5.94	6.76
S. repens	6.66 ± 0.90	23.24 ± 4.72	20.42	8.85
S. runcinata	1.79 ± 0.43	22.00 ± 3.75	76.01	6.18
S. schlechteri	nd	28.16 ± 4.69	nd	4.83
S. stenophylla	1.98 ± 0.47	12.12 ± 2.02	68.71	11.22
S. verbenaca	nd	20.85 ± 2.59	nd	6.53
5´-FU		136.06 ± 16.60		

nd: not determined, essentialoil not available

* no significant difference between the oil and the solvent extract ($P > 0.05$)

SI_1: toxicity ratio of 5´-FU over essential oil sample

SI_2: toxicity ratio of the 5´-FU over solvent extract sample

The essential oil of S. *radula* displayed the lowest toxicity profile ($P < 0.05$) (Table 11.1). An ANOVA showed no variation in the toxicity profile of *S. albicaulis*, *S. lanceolata* and

S. muirii (P > 0.05). Similarly, *S. africana-lutea*, *S. dolomitica*, *S. repens* and *S. chamelaeagnea* exhibited the same toxicity profile (P > 0.05).

The toxicity of the solvent extracts ranged from 12.12 ± 2.02 to 53.34 ± 3.90 µg/ml. The Student t-tests showed that the solvent extracts of *S. africana-caerulea* and *S. stenophylla* were similar (P > 0.05) and were the most toxic, while *S. disermas* was the least toxic relative to the other extracts (P < 0.05). An ANOVA showed that the solvent extracts of ten species namely *S. africana-lutea*, *S. aurita*, *S. chamelaeagnea*, *S. lanceolata*, *S. namaensis*, *S. radula*, *S. repens*, *S. runcinata*, *S. schlechteri* and *S. verbenaca* was not statistically different from each other (P > 0.05).

Nearly all the essential oils were significantly more toxic than their corresponding solvent extracts (P < 0.05) (Figure 11.1). However, *S. radula* showed no statistical difference between the toxicity profile of its oil and solvent extract even though the toxicity of the extract was slightly higher than that of the essential oil (Figure 11.1; Table 11.1).

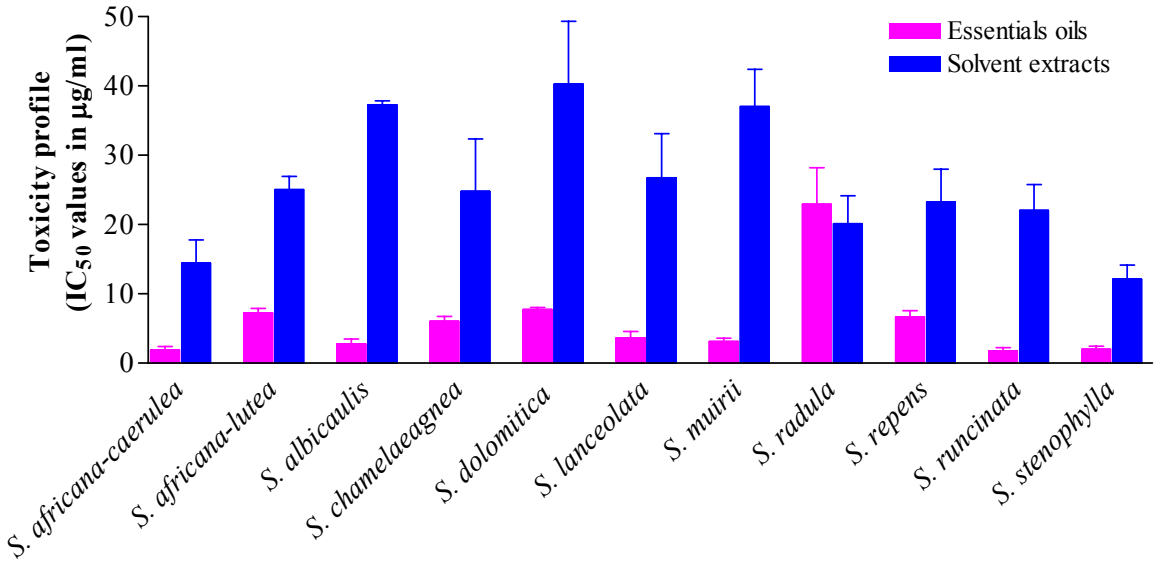


Figure 11.1 Variation between the toxicity profile of the essential oil and solvent extracts of eleven *Salvia* species (mean ± s.d., *n* = 3).

The toxicity of the essential oils and solvent extracts was compared to that of 5′-FU (safety index; SI_1 and SI_2, respectively) (Table 11.1). The standard 5′-FU was significantly less toxic compared to the essential oils and solvent extracts (P < 0.05). The solvent extracts were 3 to 11 times more toxic than the reference compound, whilst the essential oils were 6

to 76 times more toxic (Table 11.1). More precisely, the essential oil of *S. runcinata*, the most toxic essential oil (IC_{50} value: 1.79 ± 0.43 µg/ml) was 76 times more toxic than 5′-FU and the oil of *S. radula* (IC_{50} value: 22.90 ± 0.43 µg/ml) seven times more toxic than 5′-FU.

A poor correlation was observed between the anticancer activity and the toxicity profile of the solvent extracts (r = 0.37 and 0.07 against the MCF-7 and HT-29 cell lines, respectively). It may be assumed that compounds responsible for the inhibition or death of cancer cells are not necessarily those involved in the toxicity of the plants. This can also be seen by the IC_{50} value difference of 5′-FU which is about 20 times more effective in inhibiting the HT-29 cancer cells.

The isolated compounds, namely betulafolientriol oxide and salvigenin from *S. radula* (Chapter 8), carnosol from *S. chamelaeagnea* (Chapter 9) and standards of ursolic and oleanolic acids, exhibited poor toxicity against the human kidney epithelial cells with all IC_{50} values greater than 100 µg/ml.

11.5 Discussion

Plants used in traditional medicine are generally non-toxic at the administered dosage. However, it is known that all plants are toxic at certain concentrations (Liu, 2005). The essential oils and the solvent extracts displayed some degree of toxicity, with the toxicity of the essential oils being greater than that of the solvent extracts (Table 11.1). This higher toxicity of the essential oil is due to the fact that several essential oil compounds have highly hydrophobic in nature. Their accumulation in the cellular membrane disrupts the membrane, resulting in increased permeability and eventually cell death (Sikkema *et al*., 1995). *Salvia* species are not known to be toxic (Gali-Muhtasib *et al*., 2000). However, most of the tests done on the toxicity of these species used *in vivo* models (rats) rather than *in vitro* models, as is the case in the present investigation. The solvent extracts were generally less toxic than the essential oils in this study. In traditional medicine, aqueous extracts are used and a small amount of volatile fractions (containing generally non-polar compounds) would probably be extracted in hot water extracts. However, if cold water is used, essential oils will not be extracted. Furthermore, the percentage of essential oil

present in the plant is very low (Figure 4.2) and this will probably have little influence on the decoction of plant extracts as prepard traditionally.

There are various types of toxic compounds in plants and these include tannins, glycosides and alkaloids. The identification of tannins in *Salvia* species has not yet been reported in the literature, but alkaloids and glycosides are generally found in the genus (Lu and Foo, 2002). Many alkaloids are poisonous to both animals and humans. Some of the species investigated, such as *S. chamelaeagnea*, *S. namaensis* and *S. runcinata,* tested positive for alkaloids (Raffauf, 1996). The presence of these compounds was not confirmed, however if present, they may contribute to the toxicity of the solvent extracts.

The essential oils displayed greater toxicity than the solvent extracts (Table 11.1) and the toxicity of the essential oils is well reported in the literature (van Zyl and Viljoen, 2003; Nyiligira, 2004; van Vuuren *et al*., 2006). Foray *et al*. (1999) investigated the *in vitro* cytotoxicity of three essential oils of *Salvia lavandulifolia*, *S. officinalis* and *S. sclarea* against various cell lines. All three essential oils were toxic with IC_{50} values ranging from 6.3×10^{-5} to 4.4×10^{-1} µg/ml. The toxicity of the essential oils found in this study is higher than that found in other oils such as *Helichrysum picardi* (IC_{50} value: 30 µg/ml) (De La Puerta *et al*., 1993). However, these results are comparable to, and occasionally lower than essential oils from *S. lavandulifolia*, *S. officinalis* and *S. sclarea* (IC_{50} value < 0.44 µg/ml), cinnamon (IC_{50} value: 3.75 µg/ml) and clove (IC_{50} value: 7.31 µg/ml) (De La Puerta *et al*., 1993). It is difficult to deduce the constituent(s) of an individual oil that may contribute to its toxicity. In a study of 20 essential oil constituents, van Zyl *et al*. (2006) demonstrated that limonene, nerolidol, *p*-cymene, α-thujene, β-thujene, δ-terpinene and α-bisabolol displayed some degree of toxicity (IC_{50} value ≤ 10 µg/ml) in comparison to chloroquine diphosphate and quinine sulphate (two antimalarial drugs) and the 5´-FU tested in this study. Nerolidol was also found to be toxic to kidney cells (van Zyl and Viljoen, 2003). In addition, camphor is known to be highly toxic if used in prolonged treatment or when ingested in small amounts. A survey of literature conducted by Burkhard *et al*. (1999) showed that the essential oils of 11 plants, including *Salvia* were powerful anticonvulsants due to their terpenic and ketone contents. The toxicity associated with the *Salvia* essential oils may be due to the presence of the above compounds, even if present in relatively small amounts. The essential oil of *S. runcinata* was most toxic probably due to the high amount of α-bisabolol (65%, Table 4.1). Some compounds are present in relatively high amounts:

α-thujene (4.7% in *S. repens*); camphor (8.3% in *S. muirii*) and *p*-cymene (9.5% in *S. repens*). It is also possible that other compounds present even in small amount may act in a synergistic or additive manner to increase the toxicity of the essential oils (van Zyl, *pers comm*).

Although the anticancer activity and the toxicity profile measures cell viability or inhibition, a poor correlation between the anticancer activity and the toxicity of the extracts was observed. It was, however, interesting to note that with *S. lanceolata*, *S. radula* and *S. stenophylla,* which displayed anticancer activity, also exhibited some degree of toxicity. In these cases, it could be speculated that the same compound(s) may be responsible for the anticancer activity and the "general" toxicity profile of the solvent extracts. The isolated compounds from *S. radula* (betulafolientriol oxide and salvigenin, Chapter 8) and *S. chamelaeagnea* (carnosol, Chapter 9), standards of ursolic acid and oleanolic acid demonstrated poor toxicity towards the human kidney epithelial cells with IC_{50} values greater than 100 µg/ml. Thus, the activity of these compounds cannot be correlated to the overall cytotoxic profile of the *Salvia* extracts.

11.6 Conclusions

- The essential oils and solvent extracts displayed some degree of toxicity (compared to the anticancer drug, 5′-fluorouracil) with the toxicity of the essential oils being generally greater than that of the solvent extracts.
- The compounds responsible for the anticancer activity are not necessarily those responsible for the toxicity profile of the solvent extracts of *Salvia*.
- *Salvia* species are used in traditional medicine as acqueous extracts, and water will extract negligeable amount of essential oils which showed some degree of toxicity.

Chapter 12: Seasonal Variation in Essential Oil Composition and Biological Activities of Three *Salvia* Species

Abstract

Aromatic plants contain both volatile and non-volatile fractions and the chemical composition of these two fractions may be influenced by seasonal changes. The essential oil composition of *S. africana-caerulea*, *S. africana-lutea* and *S. lanceolata,* collected at the same locality throughout the 2004/2005 growing season, was compared in terms of essential oil yields, chemical composition and the biological activities. Mostly quantitative, rather than qualitative variation was observed in the essential oil composition of each species. Major fluctuations in *S. africana-caerulea* oil composition include limonene (1.5-32.6%) and viridiflorol (1.6-22.8%). Levels of α-pinene (0.5-11.9%), myrcene (2.2-11.5%) and α-eudesmol (0.1-12.9%) fluctuated seasonally in *S. africana-lutea* oil. In *S. lanceolata*, considerable changes were noted for β-caryophyllene (trace-12.9%), β-caryophyllene oxide (1.4-21.2%) and ledol (trace-11.7%). *S. lanceolata* harvested in winter was more active in winter against Gram-positive bacteria. *S. africana-caerulea* exhibited the most favourable antimalarial activity when harvested in winter (IC_{50} value: 11.61 μg/ml) which contrasts with the lowest antimalarial activity of *S. lanceolata* obtained at the same period (IC_{50} value: 42.60 μg/ml). The anti-oxidant activity of the solvent extracts also displayed variation over seasons with winter collection giving the most favourable anti-oxidant activity with *S. africana-lutea* (IC_{50} value: 10.05 μg/ml). All the solvent extracts prepared from samples collected in winter exhibited the lowest toxicity profile ($29.89 < IC_{50}$ value < 60.29 μg/ml), while the three essential oils obtained from autumn collection were more toxic ($0.03 < IC_{50}$ value < 0.4 μg/ml).

12.1 Introduction

Biological activities are correlated to the presence of chemical compounds (particularly secondary metabolites). The presence of these compounds may assist in predicting some traditional uses of medicinal plants (Rasoanaivo and Ratsimamanga-Urverg, 1993). Many plants concentrate certain secondary metabolites in specific organs resulting in the variation in biological activities between different parts of the same plant (e.g. barks, roots, leaves, stems) (Houghton, 1999). The season and even the number of hours plants receive sunlight per day, may influence the phytochemistry of the plant because some compounds may be accumulated at a particular period to respond to environmental changes (Koenen, 2001). A collection of plant material made at different times of the year may contain other possibly novel compounds with certain bio-activities (Eloff, 1999). This suggests that the biological activities should be evaluated at different times in order to determine the period of the year a plant exhibits its best activity.

A quick and effective way to assess variations in the chemical composition of plant extracts is TLC (Wagner and Bladt, 1996). While TLC can also be used to evaluate the variation in chemical composition of the essential oils, GC and GC-MS are preferred because they are not just qualitative (as with the TLC method), but also quantitative. Chemical data obtained by TLC, GC and GC-MS are important in addition to morphological features in taxonomic studies as they re-enforce the relationships of plants at a different level (McGaw *et al*., 2002).

The objectives of this study were to:

(i) determine the monthly variation in the essential oil composition of three randomly selected species,

(ii) create finger-prints using TLC for the three species, which could generate information on the chemical constituents of the solvent extract of each plant over the seasons,

(iii) evaluate the antibacterial, antimalarial and anti-oxidant activity of the solvent extracts over the seasons, investigate the toxicity profile of the essential oils and solvent extracts over seasons and determine if there is a correlation between the toxicity of the essential oils and their major compounds, and

(iv) recommend the best time of the year for harvesting of plants in terms of essential oil yields, biological activities and toxicity profile.

12.2. Materials and methods

12.2.1 Plant material

Three randomly selected species of *Salvia,* namely *Salvia africana-caerulea*, *S. africana-lutea* and *S. lanceolata*, all collected from the South Western Cape (Table 2.1), were investigated. The aerial parts of each species were harvested at the end of each month for 12 consecutive months (November 2004 to October 2005) at the same location.

12.2.2 Isolation of the essential oils and preparation of the solvent extracts

The essential oils were isolated by hydrodistillation and the solvent extracts prepared with methanol:chloroform (1:1) as previously described (Section 2.3.1 and Section 2.3.2, respectively).

12.2.2.1 Analysis of the essential oils

The essential oils were analyzed by GC and GC-MS (Section 4.2 and Section 4.3, respectively).

12.2.2.2 TLC fingerprint

To evaluate the possible impact of seasonal change on the phytochemistry, 5 µl (2 mg/ml) of the methanol:chloroform extract was separated by TLC (Alugram® Sil G/UV_{254}) and developed using ethyl acetate:toluene:acetic acid (10:5:0.01). The separated spots on TLC were examined under UV light (254 and 366 nm) and also sprayed with 0.5% anisaldehyde sulfuric acid reagent in glacial acetic acid, methanol, concentrated sulfuric acid (10:85:5) and heated at 105 ºC for five minutes.

12.2.3 Evaluation of the biological activities

The biological activities of the solvent extracts evaluated include the antibacterial, antimalarial and anti-oxidant activities. The toxicity profile was also assessed. These

activities were determined at the end of each season of the year as follows: November (spring), February (summer), May (autumn) and August (winter). The results of the biological activities corresponding to the spring samples are those reported in previous Chapters.

12.2.3.1 The antibacterial activity

The MIC values of the plant extracts over seasons was determined against Gram-positive *B. cereus* and *S. aureus* and Gram-negative *E. coli* and *K. pneumoniae* test organisms using the micro-dilution method (Section 9.2.4.1).

12.2.3.2 The antimalarial activity

The antimalarial activity was assessed using the [^{3}H]-hypoxanthine radiometric method (Section 8.2.5).

12.2.3.3 The anti-oxidant activity

The effect of seasonal change on the anti-oxidant activity of the three species was evaluated using the DPPH method as previously described (Section 6.2.2.2).

12.2.3.4 The toxicity profile

The effect of seasonal variations on the toxicity profile of the solvent extracts and essential oils was performed on human kidney epithelial cells using the MTT proliferation colourimetric method (Section 11.2.4.3).

12.3. Data analysis

The identity of the components of each oil was assigned by comparison of their retention indices, relative to C_8-C_{17} *n*-alkanes and GC-MS spectra with corresponding data of components of reference oils, laboratory-synthesized components and commercially available standards from a home-made library. Comparison of biological activities between seasons was determined using ANOVA. The IC_{50} values are given as a mean ± s.d. of at least three replicate experiments. Pearson's correlation coefficient was used to determine any correlation between the toxicity profile and major components of each essential oil.

12.4 Results

12.4.1 Variation in yield and essential oil composition

The essential oil yield of each species varied with the respective seasons. The highest yield was obtained from plants collected in late winter for *S. africana-caerulea* (August), spring for *S. africana-lutea* and *S. lanceolata* (October and September, respectively) (Figure 12.1). All three species demonstrated the lowest essential oil yield in early or mid-winter (July for *S. africana-caerulea* and *S. africana-lutea* and June for *S. lanceolata*).

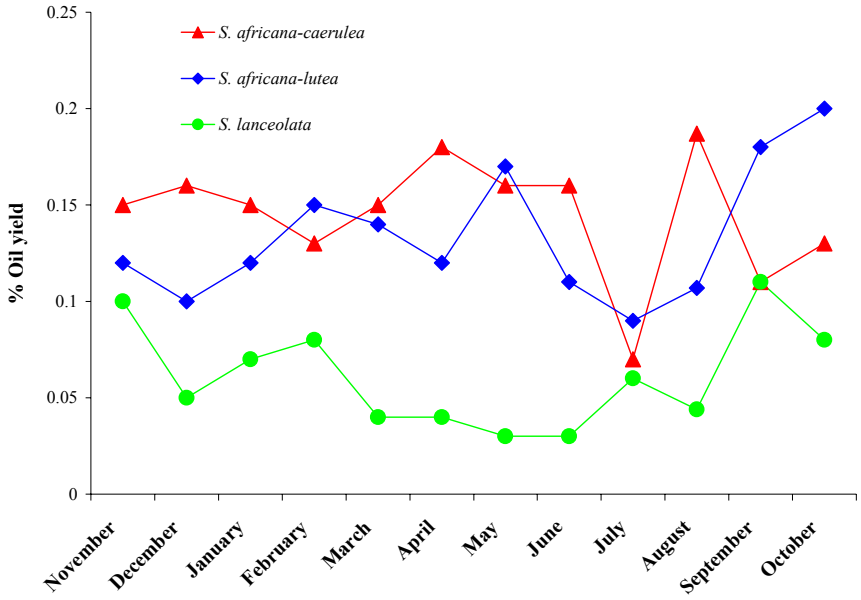


Figure 12.1 Variation in essential oil yields of three *Salvia* species over a one year period.

The components of essential oil found in each species at different seasons are those reported in Table 4.1. However, quantitative variation in the components of individual oil was noted (Appendix F). Only components of the oils greater than 4% of the total oil at least in one month are reported here. Major fluctuations in components of *S. africana-caerulea* includes α-pinene (trace-8.2%), limonene (1.50-32.6%), terpinen-4-ol (0.50-8.3%), *trans*-calamenene (0.2-8.9%) and viridiflorol (1.60-22.8%) (Figure 12.2). In the November sample of *S. africana-caerulea*, spathulenol and β-caryophyllene oxide were present in high amounts but were totally absent during the rest of the year. The components reported represent up to 60% of the total oil in January and only 20% of the total oil in December (Figure 12.2). The identified compounds of *S. africana-lutea* over a one year period are displayed in Appendix F (Table F-1).

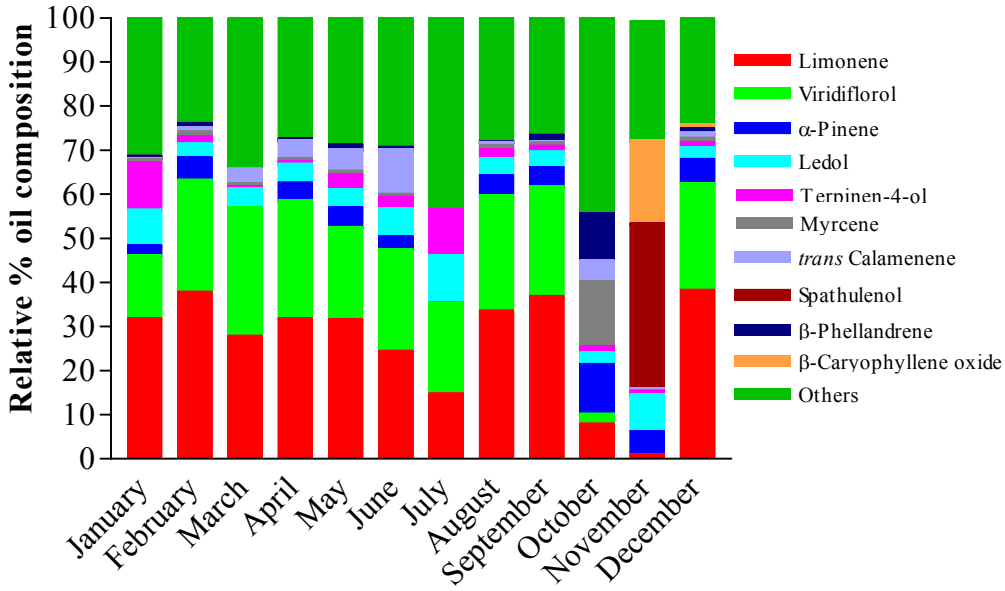


Figure 12.2 Variation of major components of *Salvia africana-caerulea* essential oil over a one year period.

The major variations in *S. africana-lutea* essential oil includes α-pinene (0.5-11.9%), myrcene (2.2-11.5%), *o*-cymene (0.4-10.3%), spathulenol (1.9-10.9%) and α-eudesmol (0.1-12.90) (Figure 12.3). These five components of *S. africana-lutea* oil represent up to 45% of the total oil in May and just below 20% in November (Figure 12.3). The identified compounds of *S. africana-lutea* over a one year period are displayed in Appendix F (Table F2).

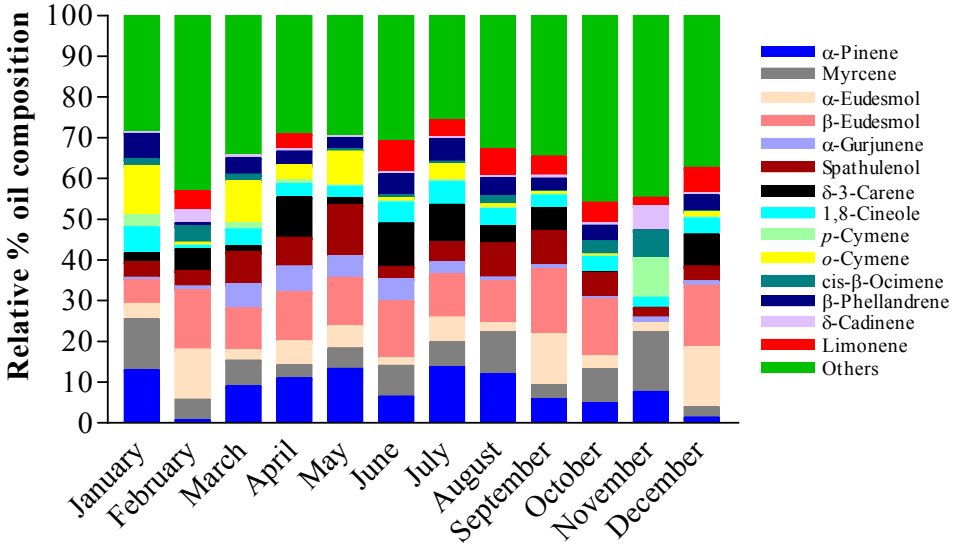


Figure 12.3 Variation of major components of *Salvia africana-lutea* essential oil over a one year period.

The major components in *S. lanceolata* showing considerable changes include β-caryophyllene (trace-12.90%), α-humulene (0.1-10.0%), spathulenol (0.7-10.6), β-caryophyllene oxide (1.4-21.2%) and ledol (0.9-11.70) (Figure 12.4). The components of *S. lanceolata* oil reported here represent close to 50% of the total oil in April and only 15% in December (Figure 12.4). The identified compounds of *S. africana-lutea* over a one year period are displayed in Appendix F (Table F-3).

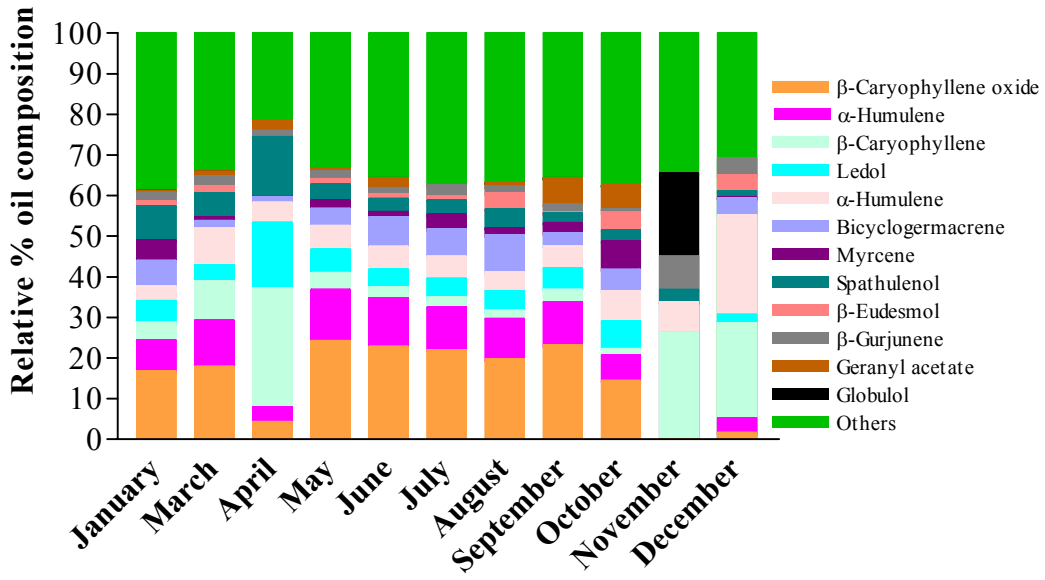


Figure 12.4 Variation of major components of *Salvia lanceolata* essential oil over a one year period.

12.4.2 TLC finger print of the solvent extracts over seasons

Like the essential oil composition, the TLC chromatograms of the solvent extracts showed a similar range of compounds for each extract prepared from plant material collected in different seasons of the year (Figure 12.5). The sample collected in the spring, however showed less compounds. Some compounds which cannot be seen in the spring sample include A, B and C (*S. africana-caerulea*), compound D (*S. africana-lutea*) and compound E and F (*S. lanceolata*).

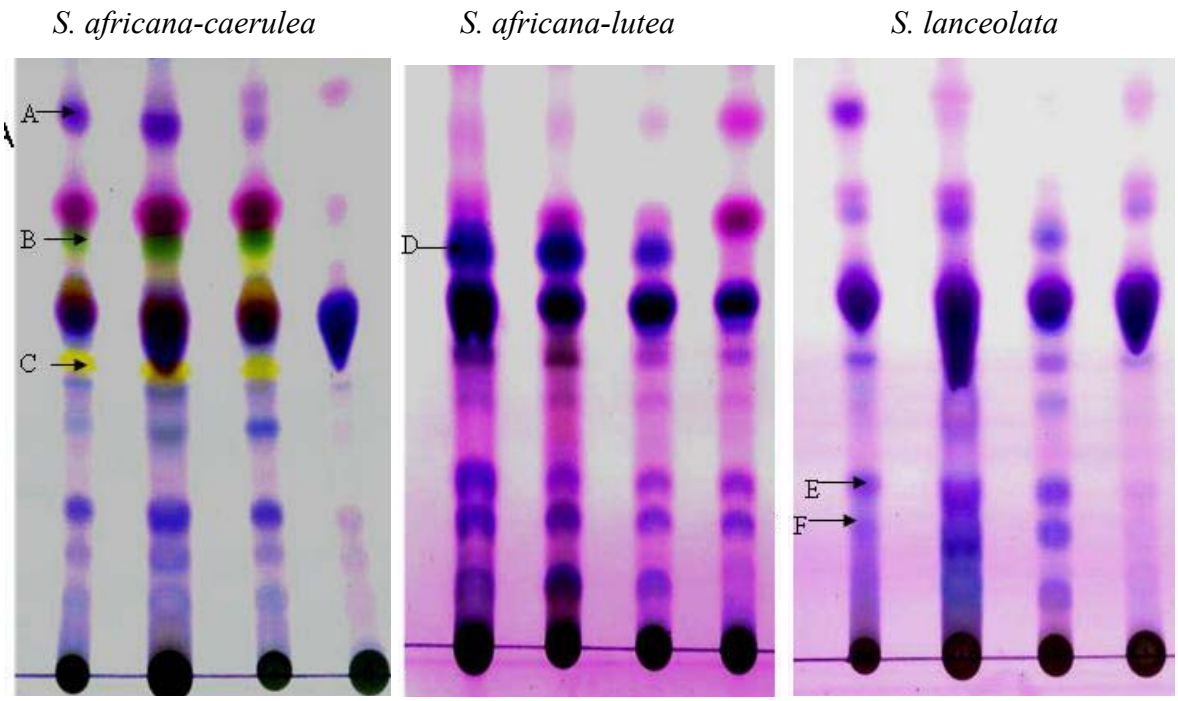


Figure 12.5 TLC chromatograms of the solvent extracts of three *Salvia* species collected at various seasons of the year: compounds A, B, C, D, E and F are those absent in the samples collected during spring.

12.4.3 Seasonal variation in biological activities

12.4.3.1 The antibacterial activity

The MIC values of each plant across seasons are given in Figure 12.6. The best activity against Gram-negative bacteria was obtained from the spring sample (November), with the exception of *S. africana-caerulea* against *K. pneumoniae*. With the Gram-positive bacteria, the best activity was exhibited against *B. cereus* from the sample harvested in spring and *S. aureus* from the collection done in autumn, respectively for *S. africana-lutea*. The solvent extract of the winter collection of *S. lanceolata* was more active (*S. aureus*) and the same activity was obtained against *B. cereus* in the collection done in summer and winter. *S. africana-caerulea* exhibited its best activity against *B. cereus* in winter and *S. aureus* in summer (Figure 12.6).

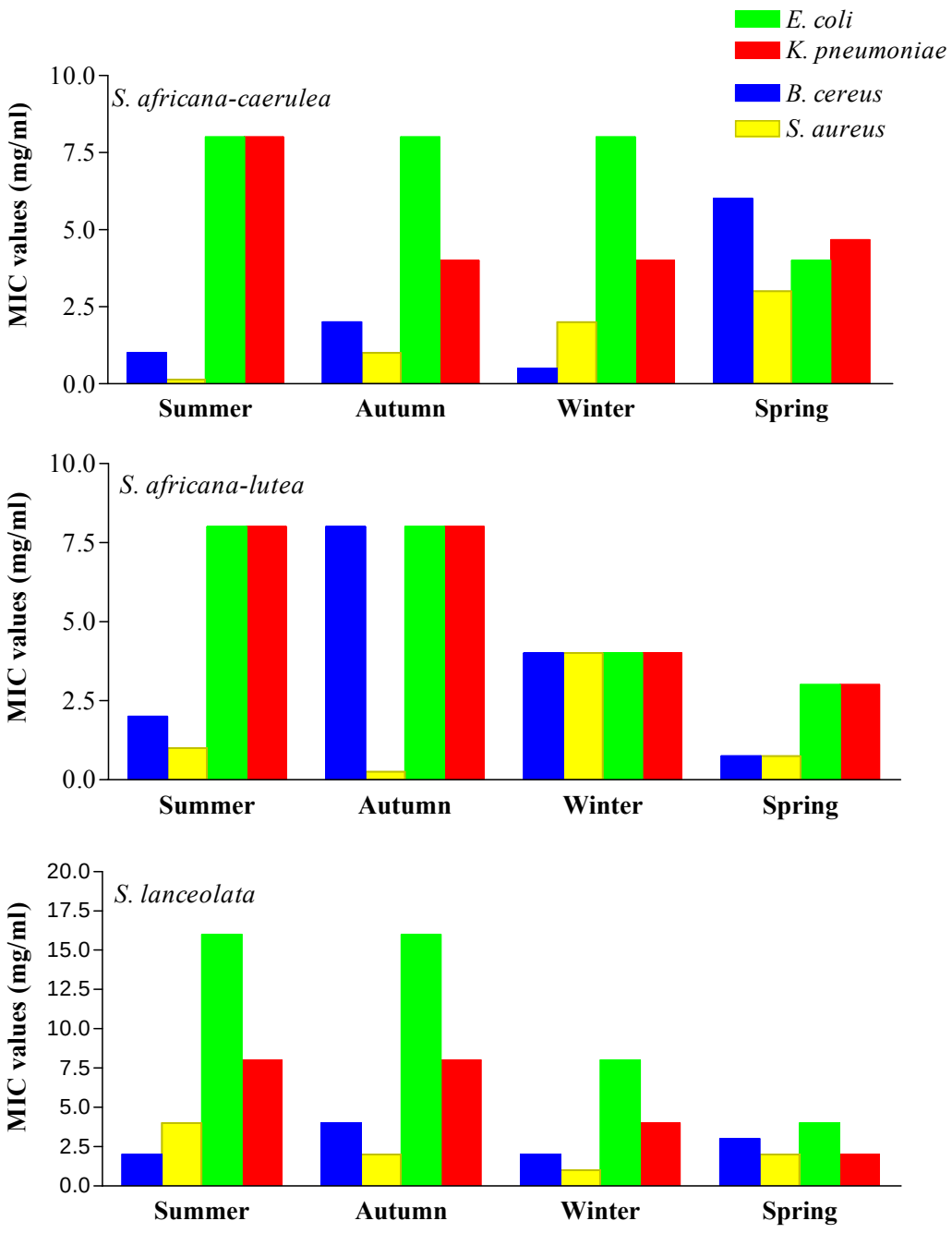


Figure 12.6 Antibacterial activity of the solvent extracts of three *Salvia* species obtained across four seasons of the year (n = 3).

12.4.3.2 The antimalarial activity of the solvent extracts

The variations in antimalarial activity of *Salvia africana-caerulea*, *S. africana-lutea* and *S. lanceolata* as influenced by the seasons are displayed in Figure 12.7. An ANOVA showed significant variation between the antimalarial activities of samples collected at different seasons ($P < 0.05$). The multiple comparison tests (Tukey test) showed that the activity of *S. africana-caerulea* from the sample collected in winter was the most favourable (IC_{50}

value: 11. 61 ± 4.53 µg/ml), whilst the autumn sample exhibited the lowest activity (IC_{50} value: 26.41 ± 3.17 µg/ml) (Figure 12.7). The activity of *S. africana-lutea* also varied over seasons with the spring sample giving the most favourable activity (IC_{50} value: 15.86 ± 5.04 µg/ml) and winter the lowest activity (IC_{50} value: 32.86 ± 2.01 µg/ml). In contrast to *S. africana-caerulea,* which exhibited its best activity with sample collected in winter, the lowest activity of *S. lanceolata* was obtained with plant harvested in winter (IC_{50} value: 42.60 ± 6.34 µg/ml), while no variation was observed between the antimalarial activity of *S. lanceolata* in spring, summer and autumn ($P > 0.05$) (Figure 12.7).

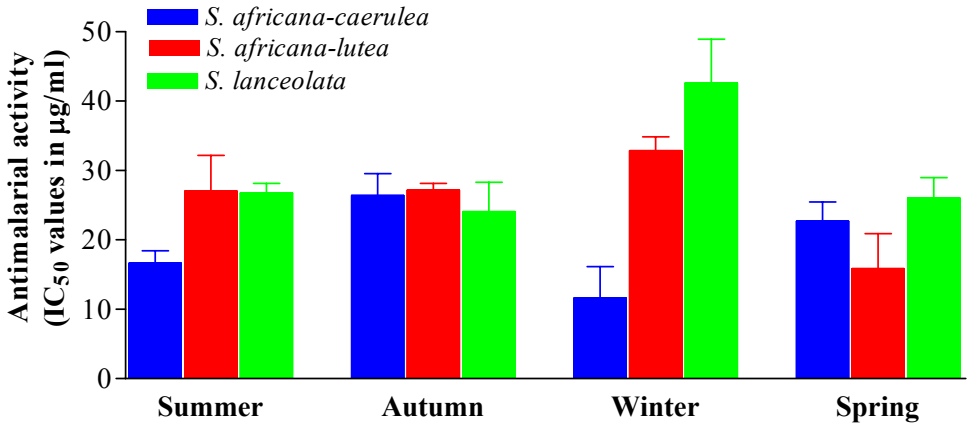


Figure 12.7 Antimalarial activity of three *Salvia* species obtained at different seasons of the year (mean ± s.d., $n = 3$).

12.4.3.3 The anti-oxidant activity of the solvent extracts

The anti-oxidant activity of the three species over four seasons is presented in Figure 12.8. An ANOVA showed significant variation in the anti-oxidant activity of each species across seasons ($P < 0.05$). The multiple comparison tests (Tukey test) showed that the activity of *S. africana-caerulea* obtained in autumn was statistically higher compared to the activity obtained in other seasons ($P < 0.05$). *S. africana-lutea* exhibited its best activity with the extract harvested in winter, which was statistically higher than that of other seasons ($P < 0.05$). In contrast, the best activity of *S. lanceolata* was obtained in summer ($P < 0.05$) with the spring extract yielding the lowest activity (Figure 12.8).

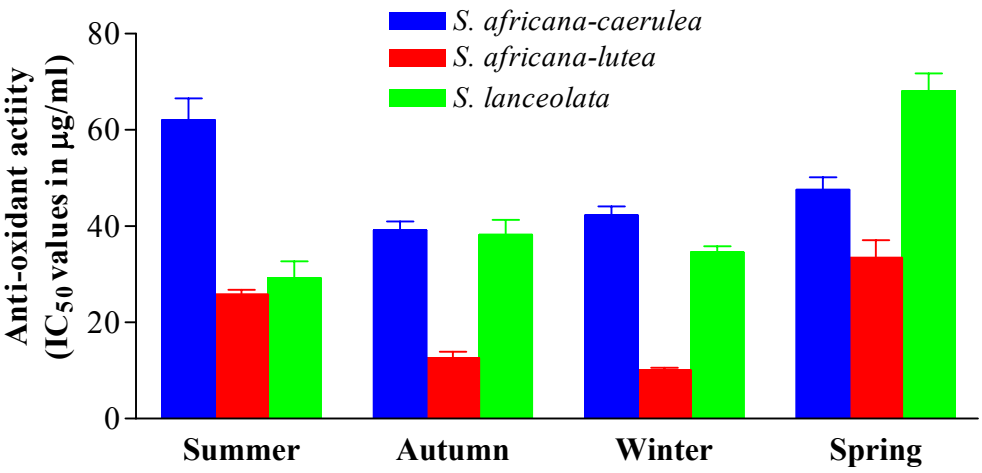


Figure 12.8 Anti-oxidant activity of three *Salvia* species obtained at different seasons of the year (mean ± s.d., $n = 3$).

12.4.3.4 Seasonal variation in the toxicity profile of the solvent extracts and their essential oils

The toxicity of the essential oils and solvent extracts was evaluated against human kidney epithelial cells. The analysis of variance showed variation in the toxicity over the four seasons, both for the solvent extracts and essential oils ($P < 0.05$) (Figure 12.9 and 12.10). The solvent extracts exhibited the lowest toxicity for all three species with sample harvested in winter (higher IC_{50} values) (Figure 12.9), while the highest toxicity profile (lower IC_{50} values) was obtained with spring sample for *S. africana-caerulea* and summer samples for *S. africana-lutea* and *S. lanceolata*.

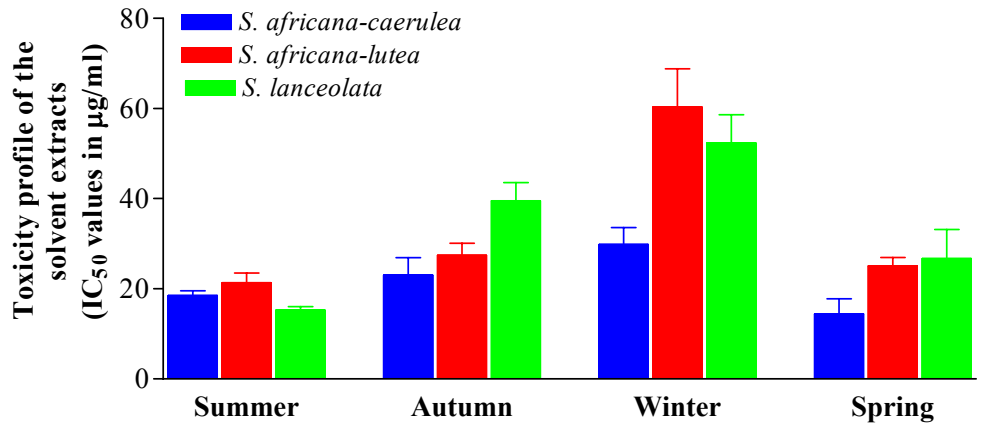


Figure 12.9 Toxicity profile of the solvent extracts of three South African *Salvia* species across seasons (mean ± s.d., $n = 3$).

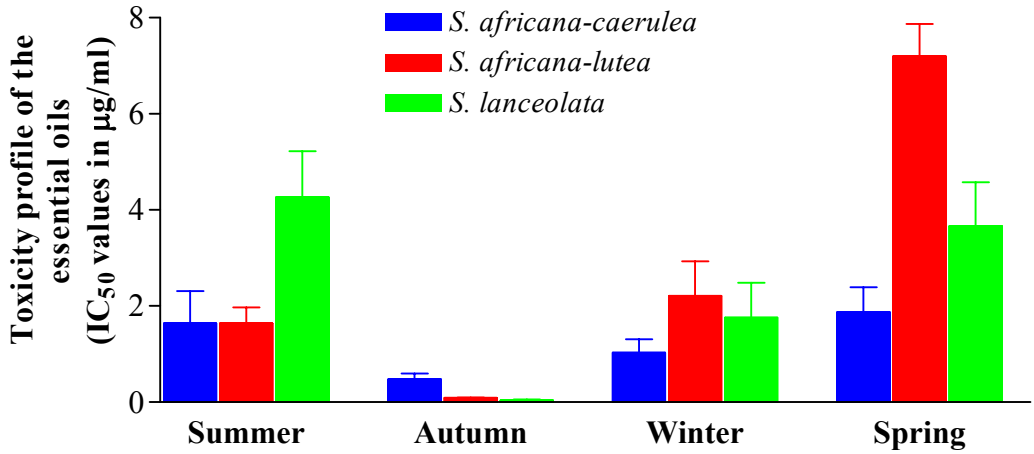


Figure 12.10 Toxicity profile of the essential oils of three South African *Salvia* species across seasons (mean ± s.d., $n = 3$).

The oils samples of the three species collected during autumn were significantly more toxic compared to other seasons. The lowest toxicity of the essential oil was obtained with samples collected during spring for *S. africana-caerulea* and *S. africana-lutea,* and sample harvested in summer for *S. lanceolata* (Figure 12.10).

12.5 Discussion

The essential oil yields varied greatly for the same species collected at different periods of the year (Figure 12.1). However, a continuous increase in the yield was noted for all species during summer (December to February, with exception of *S. africana-caerulea*). Pitarevic *et al*. (1984) recorded variation in essential oil yield of *S. officinalis* collected in Yugoslavia in various seasons, with July (flowering period) giving the highest yield. The essential oils yield varied from 0.07% (July) to 0.19% (August) for *S. africana-caerulea*. In *S. africana-lutea*, the highest yield (0.18%) was obtained in September while July gave the lowest yield (0.09%). Finally, in *S. lanceolata*, the lowest yield was obtained in June and July (0.03%) with September giving the highest yield (0.11%). The higher essential oil yield obtained in early spring, may be explained by the fact the plants remain in flower from August to January, a period of full vegetation. During this period, plants might produce substantial amounts of essential oils in order to attract more pollinators (Palá-paúl *et al.,* 2001). The yield of *S. africana-caerulea* and *S. africana-lutea* decreased from May to July which corresponds to dry season and the period of latent vegetation.

Quantitative seasonal variation in chemical composition was found in the essential oil of each species and those showing remarkable fluctuations include α-pinene, viridiflorol, limonene, terpinen-4-ol and *trans*-calamanene in *S. africana-caerulea*. Two compounds namely, β-caryophyllene oxide and spathulenol were detected in the November sample of *S. africana-caerulea*; however were absent during the rest of the year (Figure 12.2). α-Pinene, myrcene, *o*-cymene, spathulenol and α-eudesmol showed fluctuations in *S. africana-lutea* between seasons (Figure 12.3), while β-caryophyllene, α-humulene, spathulenol, β-caryophyllene oxide and ledol showed major variations in *S. lanceolata* (Figure 12.4). Although no pattern in the fluctuation of the components of an individual oil component was observed, the most conspicuous variation was recorded for β-caryophyllene and β-caryophyllene oxide in *S. lanceolata*. The variation of these two compounds was inversely related: a rise in the β-caryophyllene oxide content was accompanied by a decrease in the content of β-caryophyllene (Figure 12.11). The content of β-caryophyllene oxide started to increase from January and reached its maximum in April when the content of β-caryophyllene was at its lowest level (Figure 12.11).

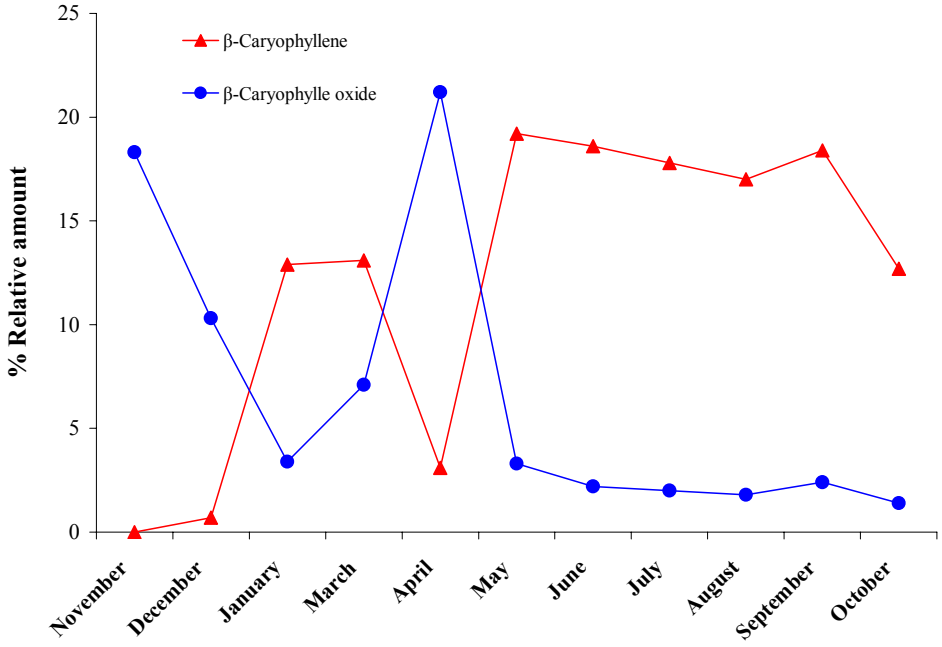


Figure 12.11 Variation in β-caryophyllene and β-caryophyllene oxide contents in *S. lanceolata* over seasons.

Researchers have reported seasonal variation in essential oil yields and composition for many species. Pitarevic *et al*. (1984) also noted variation in essential oil composition of *S. officinalis* collected at various seasons of the year. The components of the oil with significant variation included 1,8-cineole (5.8-13.2%), α-thujene (10.9-23.1%), β-thujene

(25.0-40.1%) and camphor (7.1-16.4%). The qualitative variation in essential oil composition of *S. libanotica* was also demonstrated (Farhat *et al.,* 2001). The major components of the oil including 1,8-cineole (47.7-57.4%) and camphor (7.7-12.3%) fluctuated from one season to another. The seasonal variation in the composition of the essential oil of *Crithmum maritimum* L. (Umbelliferae) also showed a great degree of variation in the essential oil composition of the main components, namely sabinene (7-42%) and γ-terpinene (26-55%) (Barroso *et al*., 1992), but the essential oil yield of this species did not show remarkable change. This study has demonstrated that the essential oil yield and change in the amount of each component varied from one season to another. It is known that many factors affect the essential oil yield and composition such as season, temperature and reproductive stage (Putievsky *et al*., 1986).

The biological activities of the same plant collected at different periods of the year showed significant variation, which could be attributed to changes in the amounts of the active compounds present in each plant in different months. Changes in chemical composition could be explained in terms of a thermoregulatory action of the hydrophobic compounds which could protect the plant from desiccation. Furthermore, polar solids (triterpenes, flavonoids, diterpene acids) present in the plants might act as a physical barrier to prevent water permeation and dehydration (Harbone *et al*., 1975).

Many studies have reported differences in the biological activity of the same plant collected in different seasons. McGaw *et al*. (2002) investigated the variation in antibacterial activity of *Schotia* species and noted slight fluctuations. They also noted small variations in the chromatograms obtained from one season to another. Maréchal *et al*. (2004) also demonstrated seasonal variation in antibacterial activity of solvent extracts of *Bifurcaria bifurcata* (Cystoseiraceae) with the highest level of activity obtained from spring to summer. In the current study, major fluctuations in the antibacterial activity of the three *Salvia* species investigated were observed. Furthermore, the variation obtained in the antibacterial activity depended on both the plant species and the test organism involved. Variation in the anti-oxidant activity and toxicity profile of the three species across seasons was exhibited. Farhat *et al*. (2001) investigated the essential oil of *S. libanotica* across four seasons of the year and found that the winter sample was the most toxic, while the spring sample was the least toxic. The toxicity of the solvent extracts of all three species was low during winter (Figure 12.9), while the three essential oils were

significantly (4 to 142 times) more toxic during autumn (Figure 12.10). An attempt to find a relationship between the amount of major components of each essential oil and the toxicity profile was not successful. Thus it can be assumed that the concentration of the essential oil components is not directly involved in the toxicity of the essential oils. To account for this, two possibilities can be proposed: either the toxicity profile was due to other minor components or there was a synergistic effect between the major components of the essential oils and other molecules. A negative correlation was observed between the toxicity and the major components [Pearson's correlation coefficient r = -0.98, $P < 0.05$, (*S. africana-lutea)*; r = - 0.97, $P < 0.05$, (*S. lanceolata*)] denoting that an increase in the amounts of the major components of the essential oil are accompanied by an increase in the toxicity. However, *S. africana-caerulea* showed no significant correlation (r = 0.45, $P > 0.05$) between the toxicity of the oil and its major components. This suggests that both major and minor components of the essential oil may interact to contribute to the toxicity of the oil. This investigation has demonstrated the importance of seasonal changes on biological activity and essential oil yield.

12.6 Conclusions

- Quatitative variation in the essential oil composition and yields exists over seasons and this also depends on the species studied.
- Variation in the chemical profile of the solvent extracts was observed on the TLC plates as for the various seasons.
- Seasonal/climatic changes play an important role in the biological activities and the toxicity of the plants.
- The best activity of the solvent extract of *S. africana-caerulea* was obtained in summer (Gram-positive), winter (antimalarial activity) and autumn (anti-oxidant activity).
- The best activity of *S. africana-lutea* was obtained in spring (antibacterial activity), spring (antimalarial activity) and winter (anti-oxidant activity).
- *S. lanceolata* exhibited the best activity in winter (against Gram-positive bacteria), spring (against Gram-negative bacteria), winter (antimalarial activity) and summer (anti-oxidant activity).

- The solvent extracts of all three species exhibited the lowest toxicity in winter, while the essential oils of the three species were less toxic in spring. The toxicity of the essential oils is not always related to presence of the major components, but the influence of minor components to act synergistically with other components needs to be evaluated to understand the overall toxicity of the essential oils.
- It is difficult to determine which season will give the best activity because this varies with the species involved as well as the biological activity studied. However, it is important to use more plants in order to have a good pattern.

Chapter 13: The Influence of Essential Oils on the Biological Activities of Solvent Extracts

Abstract

The solvent extracts of aromatic plants contain both the volatile (essential oils) and non-volatile compounds. The biological activities of the solvent extracts of three *Salvia* species (*Salvia africana-caerulea*, *S. africana-lutea* and *S. lanceolata*) were evaluated in the presence and absence of essential oils. The biological activities assessed included the antibacterial, anti-oxidant, antimalarial and anticancer activity. In addition, the toxicity profile was also investigated. The solvent extract of *S. africana-caerulea* without essential oil exhibited the best activity against Gram-positive bacteria (MIC value: 0.1 mg/ml), while the solvent extract containing essential oil of *S. africana-lutea* was the most active against Gram-negative bacteria (MIC value: 3 mg/ml). No significant difference was obtained in the antimalarial activity for the two extracts of *S. africana-caerulea* and *S. lanceolata*, while the activity of the solvent extract without essential oil was significantly higher than that of the solvent extract containing essential oil in *S. africana-lutea* ($P < 0.05$). The toxicity profile of all three species was significantly higher ($P < 0.05$) with the solvent extracts containing essential oils.

13.1 Introduction

Aromatic plants have traditionally been used in folk medicine as well as to extend the shelf life of foods. In the previous Chapters, it was observed that the essential oils and the solvent extracts of indigenous *Salvia* species exhibited a broad range of biological activities including anti-oxidant (Chapter 6), anti-inflammatory (Chapter 7), antimalarial (Chapter 8), antibacterial (Chapter 9) and anticancer (Chapter 10) activity. The essential oils and solvent extracts also displayed various levels of toxicity against normal cells with the toxicity of the essential oil being greater than that of the solvent extracts (Chapter 11). The solvent extracts contain both the essential oils (volatiles) and non-volatile compounds (e.g. phenolics). In order to investigate whether the removal of essential oils could reduce the toxicity of the solvent extract without affecting the biological properties, the investigation of the solvent extracts in the presence and absence of the essential oils was conducted.

The objective of this study was to evaluate the antibacterial, anti-oxidant, anticancer and antimalarial activities of two solvent extracts (with and without essential oils) of three *Salvia* species and to compare the toxicity of these solvent extracts.

13.2 Materials and methods

13.2.1 Plant material

Three randomly selected species namely *Salvia africana-caerulea*, *S. africana-lutea* and *S. lanceolata*, all collected from the South Western Cape (Table 2.1) were investigated.

13.2.2 Isolation of the essential oils and preparation of the solvent extracts

The essential oils were isolated by hydrodistillation as previously described in Section 2.3.1. In order to investigate the effect of the essential oils on selected biological activities, the residue of the plant material in the Clevenger apparatus (with water) was air-dried at room temperature and then extracted as described in Section 2.3.2. To provide a better understanding of the terminology used in this Chapter, the plant collected from the field and air-dried is referred to ‘as the solvent extracts containing essential oils or solvent extracts with essential oils’. The plant material remaining in the Clevenger apparatus,

which was then air-dried and also extracted with methanol:chloroform, are referred to as 'the solvent extracts without the essential oils or solvent extracts in the absence of the essential oils'. The same plant material was used and three-quarters of fresh plant material of each species was hydrodistilled and the remaining one-quarter air-dried at room temperature to constitute the extracts without essential oils and the extracts containing essential oils, respectively.

13.2.3 Comparison of the TLC plates of the solvent extract with and without essential oils

In order to verify whether the high temperature used to isolate the essential oils would affect the plant extract, a TLC plate representing the solvent extract with essential oil and solvent extract without essential oil were prepared. Hence, 5 µl (2 mg/ml) of each methanol:chloroform extract from *S. africana-caerulea*, *S. africana-lutea* and *S. lanceolata* were separated by TLC (Alugram® Sil G/UV_{254}) and developed using ethyl acetate:toluene:acetic acid (10:5:0.01). The separated spots were examined under UV light (254 and 366 nm) and also visualised by spraying the plates with 0.5% anisaldehyde sulfuric acid reagent in glacial acetic acid, methanol, concentrated sulfuric acid (10:85:5) and heated at 105 ºC for five minutes.

13.2.4 Evaluation of the biological activities

The biological activities evaluated included the antibacterial, anticancer, antimalarial and anti-oxidant activities, as well as the toxicity profile being determined for the extracts.

13.2.4.1 The antibacterial activity

The MIC values of the solvent extracts with and without the essential oils were determined against Gram-positive *B. cereus* and *S. aureus* and Gram-negative *E. coli* and *K. pneumoniae* test organisms using the micro-dilution method (Section 9.2.4.1) and compared to activity of the essential oils alone (Table 9.1).

13.2.4.2 The anticancer activity

The anticancer activity of the solvent extracts in the presence and absence of the essential oils was determined using the SRB method (Section 10.2.3) against the HT-29 and MCF-7 cells.

13.2.4.3 The antimalarial activity

The antimalarial activity of the solvent extracts with and without essential oils was assessed using the [^{3}H]-hypoxanthine radiometric method (Section 8.2.5) and compared to activity of the essential oils alone (Table 8.1).

13.2.4.4 The anti-oxidant activity

The effect of the essential oils on the anti-oxidant activity of the two solvent extracts was determined using the DPPH method as previously described (Section 6.2.2.2) and compared to activity of the essential oils alone (Table 6.1).

13.2.4.5 The toxicity profile

The toxicity profile of the two solvent extracts was determined using the MTT proliferation colourimetric method on human kidney epithelial cells (Section 11.2.4.3) and compared to activity of the essential oils alone (Table 11.1).

13.3 Data analysis

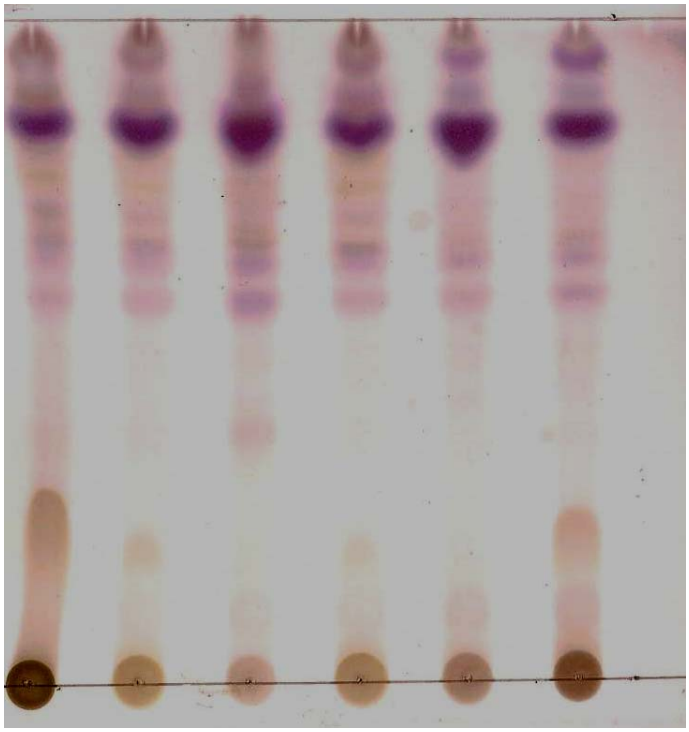
The IC_{50} values are given as a mean ± s.d. of three replicate experiments. The difference between the activity of the solvent extracts with and without essential oils was determined using the paired sample t-tests. All statistical analyses were performed using Statistica® version 5.0 software and $P < 0.05$ was considered significant.

13.4. Results

13.4.1 Comparison of the TLC plates of the solvent extracts with and without the essential oils

There was not obvious variation in the chromatographic profiles in the solvent extracts of the three species studied in the presence or absence of the essential oils (Figure 13.1).

Similar compounds were noted and it appeared that the high temperature did not alter the composition of the phenolic compounds based on TLC plate results.



SacW SacWT SalW SalWT SlaW SlaWT

Figure 13.1 TLC chromatogram of the solvent extracts of three *Salvia* species obtained in the presence and absence of the essential oils. SacW: solvent extract of *S. africana-caerulea* with essential oils; SacWT: solvent extract of *S. africana-caerulea* without essential oils, SalW: solvent extract of *S. africana-lutea* with essential oils; SalWT: solvent extract of *S. africana-lutea* without essential oils; SlaW: solvent extract of *S. lanceolata* with essential oils; SlaWT: solvent extract of *S. lanceolata* without essential oils.

13.4.2 The antibacterial activity

A comparison between the activity of the solvent extracts containing essential oils and the solvent extracts without essential oils is displayed in Figure 13.2. The best activity against Gram-positive bacteria for all three species was obtained with the solvent extracts without essential oils. In contrast, the best activity against Gram-negative bacteria was obtained with the solvent extracts containing essential oils with the exception of *S. africana-caerulea* against *K. pneumoniae* (Figure 13.2).

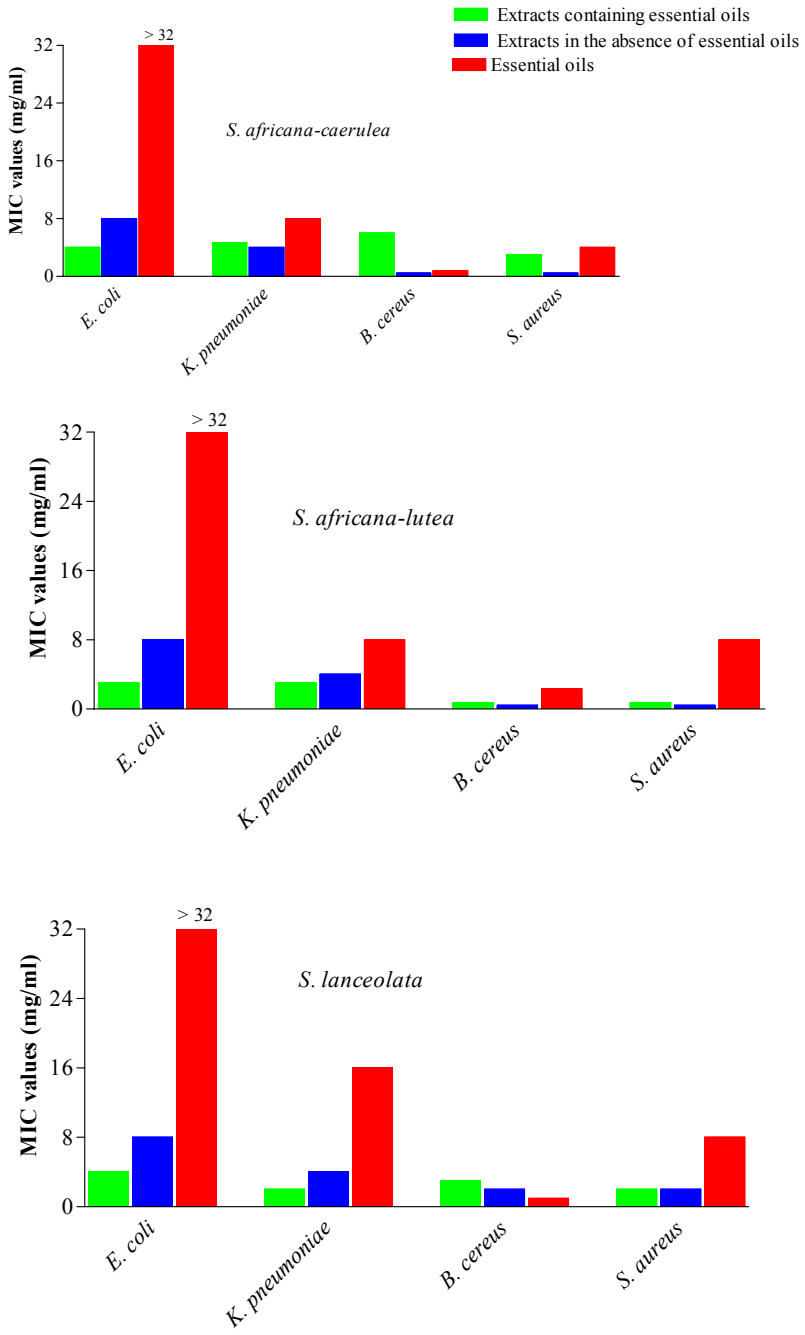


Figure 13.2 Antibacterial activity of the solvent extracts with and without essential oils of three *Salvia* species (n = 3).

13.4.3 The anticancer activity

The results of the anticancer activity of the two solvent extracts (with and without essential oils) against two cancer cell lines are displayed in Figure 13.3. A paired sample t-test showed no difference between the solvent extracts containing essential oil and the solvent extract without essential oil against the HT-29 cell line ($P > 0.05$). In *S. africana-lutea*, the solvent extract containing essential oil was significantly less active than the solvent extract

without essential oil against the MCF-7. In contrast, *S. africana-caerulea* exhibited the most favourable activity with the solvent extract containing essential oil, while in *S. lanceolata* there was no difference between the two solvent extracts against the MCF-7 cell line.

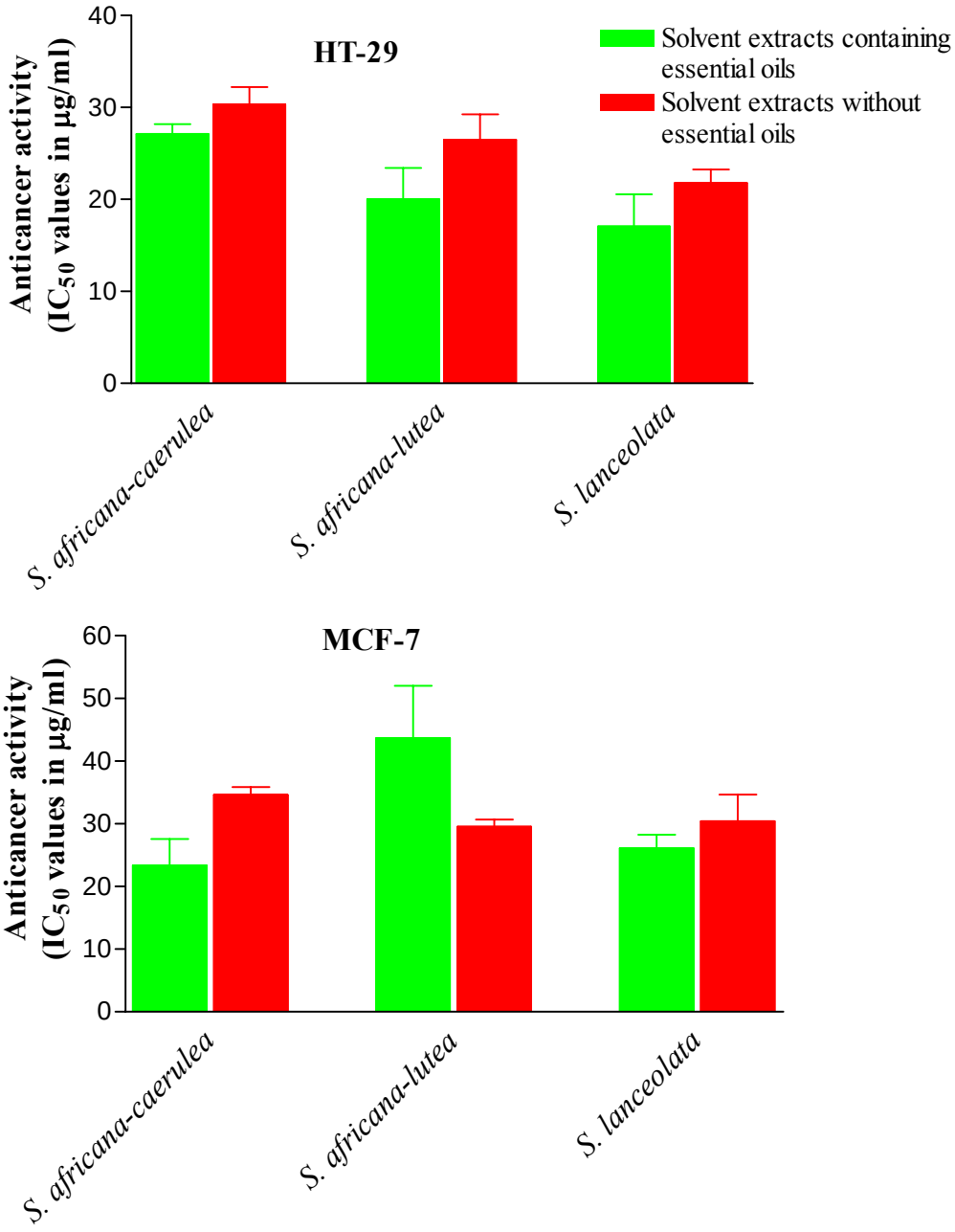


Figure 13.3 Anticancer activity of the solvent extracts of three *Salvia* species evaluated in the presence and in the absence of the essential oils (mean ± s.d., $n = 3$).

13.4.4 The antimalarial activity

The antimalarial activity of the two solvent extracts against the chloroquine-resistant *P. falciparum* strain is depicted in Figure 13.4. A paired sample t-test showed no variation in the antimalarial activity of *S. africana-caerulea* and *S. lanceolata* using the extracts with and without the essential oils ($P > 0.05$). The solvent extract of *S. africana-lutea* without essential oils exhibited higher activity ($P < 0.05$) compared to the solvent extract with essential oils (Figure 13.4). In all cases, the antimalarial activity of the solvent extracts (with and without essential oils) was significantly lower than the activity of the essential oils (Figure 13.4).

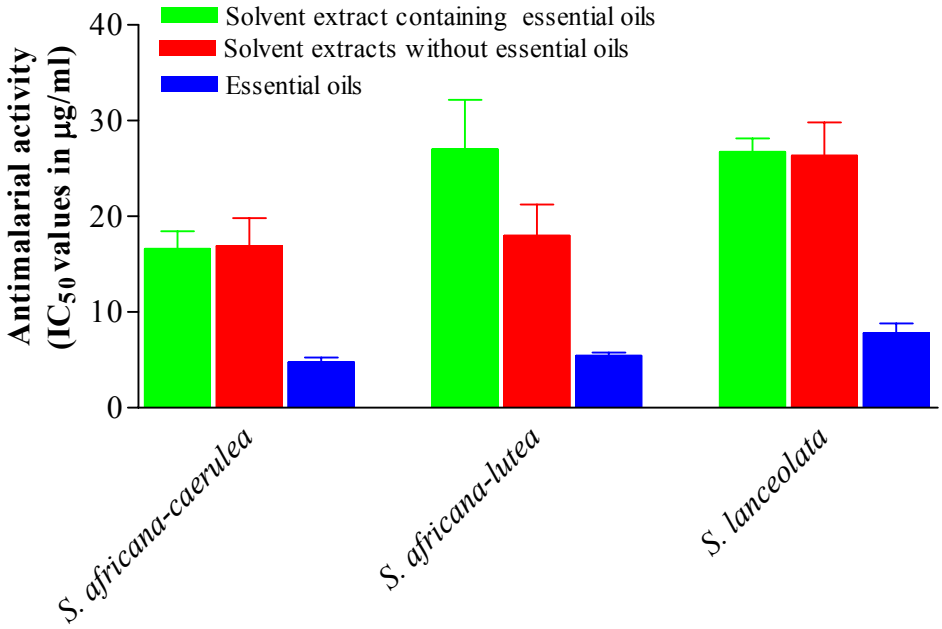


Figure 13.4 Variation in the antimalarial activity of the solvent extracts with and without their respective essential oils of three *Salvia* species (mean ± s.d., $n = 3$).

13.4.5 The anti-oxidant activity

The anti-oxidant activity of the two solvent extracts is depicted in Figure 13.5. A paired sample t-test showed that the activity of the solvent extracts containing essential oils was significantly lower ($P < 0.05$) compared to the activity of the solvent extracts without essential oils for all three species. This suggests that the essential oils contributed little to the anti-oxidant activity of the solvent extracts.

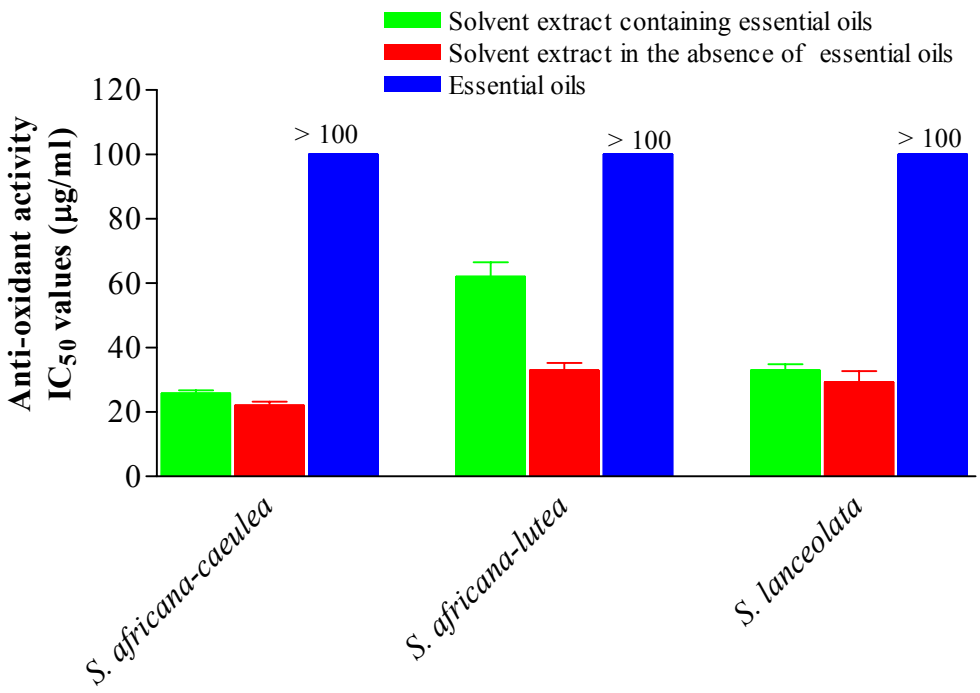


Figure 13.5 Anti-oxidant activity of the solvent extracts with and without their respective essential oils of three *Salvia* species (mean ± s.d., $n = 3$).

13.4.6 The toxicity profile

The outcome of the toxicity profile of the two solvent extracts is displayed in Figure 13.6. A paired sample t-test indicated that the toxicity of the solvent extracts was significantly lower in the absence of the essential oils ($P < 0.05$) implying that the essential oils have contributed significantly to the toxicity of the solvent extracts of these three species. The toxicity of the essential oil was always higher than that of the two solvent extracts (with and without essential oils). In order to determine whether there is a direct correlation between the toxicity of the oil and solvent extract of the same plant, a regression analysis was performed. A poor relationship ($r^2 = 0.25$) between the activity of the essential oil and the solvent extract of the same plant was observed. This suggests that the high toxicity of an essential oil does not imply that its solvent extract is also highly toxic.

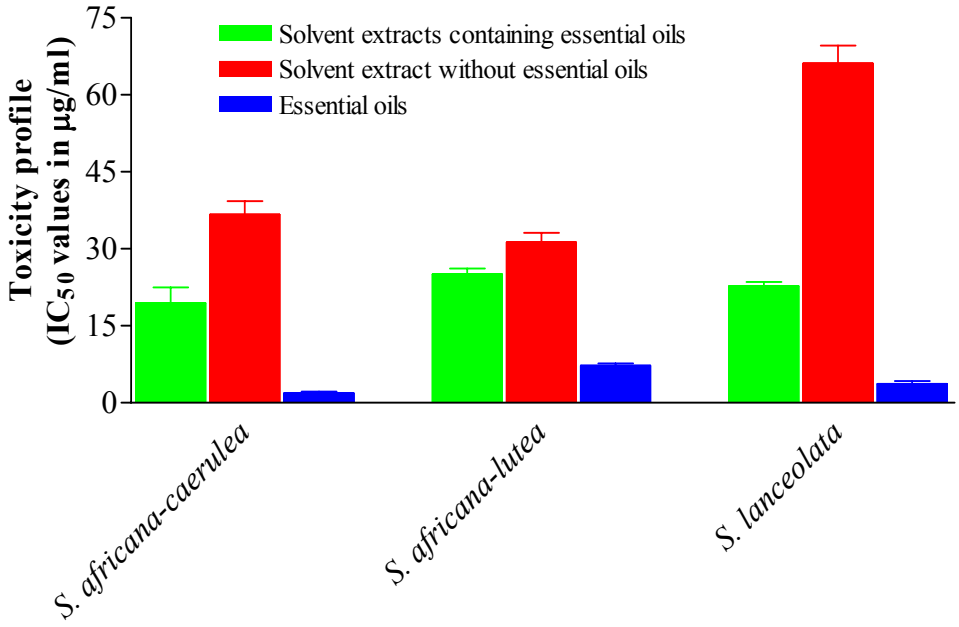


Figure 13.6 Toxicity profile of the solvent extracts of three *Salvia* species with and without essential oils (mean ± s.d., $n = 3$).

13.5 Discussion

There is a variation in the *in vitro* biological activities of the solvent extracts when tested with and without the essential oils. The essential oils seem to have beneficial or adverse effects depending on the biological activities implicated. In this study, it was found that the essential oils do not have a negative influence on the activity of the solvent extracts against Gram-positive bacteria, while they increase the activity of the solvent extracts against Gram-negative bacteria. The essential oils also slightly increase the activity of the solvent extracts against cancer cells. The toxicity of the solvent extracts was significantly decreased when the essential oils were removed implying that the essential oils contribute to the toxicity of the solvent extracts. A poor relationship obtained between the toxicity of the essential oils and the solvent extracts may be due to the fact that essential oils were present in relatively small amounts in the solvent extracts (Figure 4.2).

Although the mechanism of action of the essential oil is not clearly understood, the hydrophobic compounds present in essential oils are proposed to disrupt cell membranes resulting in increased permeability to ions which will cause cell death (Sikkema *et al*., 1995; Cowan, 1999).

Although the activity of the essential oils was generally greater than that of the solvent extracts, this study has demonstrated that the removal of the essential oils did not significantly reduce the antimalarial activity of the solvent extract (for *S. africana-caerulea* and *S. africana-lutea*) as one may have assumed. Solvent extracts contain both volatile and non-volatile fractions. In Chapter 6 it was shown that the essential oils possessed poor anti-oxidant activity (IC_{50} values > 100 μg/ml), thus it could be expected that the removal of essential oils would not affect the anti-oxidant activity of the solvent extracts. This assumption was correct as the solvent extracts still exhibited favourable anti-oxidant activity once the essential oil had been removed (Figure 13.5). It is known that there is a direct relationship between the anti-oxidant activity and the total phenolic content (Chapter 6; Cuvelier *et al*., 1994, Cuvelier *et al*., 1996; Hohmann *et al*., 1999). The dilution of total phenolic content after the removal of the essential oils affects the anti-oxidant capacity, as well as other biological activities, of the solvent extracts.

13.6 Conclusions

- The solvent extracts have varying degrees of activities, and depend on the presence or absence of essential oils.
- The solvent extracts without the essential oils exhibited the best activity against Gram-positive bacteria, while those containing essential oils displayed the most favourable activity against Gram-negative bacteria. The hydrophobic essential oil compounds may therefore be important to disrupt the cell membrane and increasing the activity of the solvent extracts.
- The presence of the essential oils in the solvent extracts may have antagonistic, synergistic or additive interactions depending on the biological activity investigated.
- The presence of essential oils in the solvent extracts reduces the concentrations of phenolic compounds which are the main contributors of the anti-oxidant activity of the solvent extracts.
- *S. africana-caerulea* and *S. lanceolata* showed no significant variation in antimalarial activity between the two extracts. However, the solvent extract of *S. africana-lutea* containing essential oil was lower than that of the solvent extract without essential oil.

- The solvent extract containing essential oils was more toxic to “normal cells” compared to the solvent extracts without essential oils.
- The essential oils contribute to the toxicity of the solvent extracts.

Chapter 14: General Conclusions and Recommendations

14.1 General conclusions

The aims of the study were to evaluate the biological activities of the solvent extracts and essential oils of indigenous *Salvia* species, to isolate and identify compounds which may be responsible for the biological activities and to determine any possible relationship between the species investigated using the essential oil composition and the leaf trichomes type data. In addition, the effect of seasons on the chemical composition of essential oil was studied as well as the influence of the essential oils on the biological activities of the solvent extracts. The aims of the study were achieved and the major findings are listed below.

14.1.1 Leaf trichome types and essential oil composition

- Both glandular (peltate and capitate) and non-glandular trichomes were observed in all the investigated species.
- The peltate trichome consists of eight to sixteen cells in the head arranged in one or two concentric circles.
- Four types of capitate trichomes were recorded based on the number of cells in the head and the length of the stalk cell and this helped to divide the investigated species into four groups.
- Three species namely *S. aurita*, *S. chamelaeagnea* and *S. repens* have multiple cells at the base of the non-glandular trichomes, while others only have one cell at the base.
- The essential oil yield of the eleven species studied was low (0.004-0.5%) and varied between species with *S. muirii* and *S. radula* having the highest and the lowest yield, respectively.
- Quantitative and qualitative variations in essential oil were recorded for the essential oils investigated.
- Ninety-three components composed mainly of monoterpene and sesquiterpene hydrocarbons, oxygen-containing monoterpenes and oxygen-containing sesquiterpenes were recorded.

- Major components identified include α-pinene, 1,8-cineole, linalool, limonene, myrcene, β-caryophyllene, spathulenol, β-caryophyllene oxide, viridiflorol, δ-3-carene and α-bisabolol.
- *S. runcinata* was found to particularly rich in α-bisabolol (65% of the total oil) and may therefore be considered as a natural source of α-bisabolol.
- Cluster analysis data revealed that the essential oils of *S. africana-caerulea* and *S. lanceolata* were highly correlated due to their high amount of spathulenol and β-caryophyllene oxide, which supports the morphological resemblance of the two species. The correlation between the essential oil of *S. dolomitica* and the rest of the species was very low.

14.1.2 Identification of phenolic compounds in the solvent extracts

- Indigenous *Salvia* species contain various phenolic compounds such as rosmarinic acid, carnosol, ursolic acid, oleanolic acid, salvigenin, 7-*O*-methylepirosmanol, betulafolientriol oxide, caffeic acid and carnosic acid.
- Betulafolientriol oxide isolated from *S. radula* was detected in all the 17 species investigated. Various populations need to be investigated in order to determine whether this compound could be used as taxonomic marker for indigenous *Salvia* species.
- Rosmarinic acid was detected in all the indigenous species, except *S. verbenaca*.
- *S. muirii*, *S. runcinata* and *S. albicaulis* have high levels of rosmarinic acid (> 35%), while the levels of carnosol and/or carnosic acid are high in *S. aurita*, *S. chamelaeagnea*, *S. namaensis* and *S. stenophylla* (> 20%).

14.1.3 Biological activities and isolated compounds from two *Salvia* species

- The solvent extracts of indigenous *Salvia* species are good anti-oxidant but exhibited poor anti-inflammatory activity.
- In contrast, the essential oils possessed favourable anti-inflammatory activity, but poor anti-oxidant activity.
- Qualitative analysis by TLC indicated that the same compounds possessed the ability to scavenge the $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ radicals.

- The anti-oxidant capacity of the *S. schlechteri* extract was higher than that of the reference compound, vitamin C which indicates that this plant may be used as Natural anti-oxidant.
- The solvent extracts and essential oils inhibited the *in vitro* growth of *P. falciparum* and in most of cases the activity of the essential oils was greater than that of its counterpart solvent extract.
- Betulfolientriol oxide and salvigenin isolated from *S. radula* exhibited comparable or lower activity than the crude extract and chloroquine and quinine. This suggests that the activity of the crude extract is determined by the combination of various compounds.
- The solvent extracts and/or essential oils displayed varing degrees of activity against bacterial, human cancer cells and human kidney epithelial kidney cells.
- The solvent extracts of *S. chamelaeagnea*, *S. disermas*, *S. dolomitica* and *S. muirii* exhibited good activities in various assays, while their toxicity to human epithelial cells were low in comparison to other solvent extracts.
- *S. africana-caerulea* was the only species displaying anticancer activity on all three human cancer cell lines with an IC_{50} less than 30 µg/ml.
- The antibacterial and anticancer activities of the essential oils and/or solvent extracts against the test patogens provide a scientific rationale of the use of the taxa in traditional herbal remedies for the treatment of respiratory ailments, dermal infections, tuberculosis and some cardio-vascular diseases.
- The *in vitro* combination of *S. chamelaeagnea* and *L. leonurus* demonstrated a synergistic interactions against *B. cereus* and *S. aureus*, providing support for the co-administration use of these two species.
- Carnosol, ursolic acid/oleanolic acid, 7-*O*-methylepirosmanol were isolated from *S. chamelaeagnea* and tested against bacterial pathogens. Their activities were in most cases higher than that of the crude extract, but less active than ciprofloxacin.
- No direct correlation could be observed between the chemical composition of the essential oils and the solvent extracts and the biological activities. Hence, the activity of the essential oil or solvent extract is most likely the result of the combination between the major and minor compounds.

14.1.4 Seasonal variation in essential oil composition and biological activities and the influence of the essential oil in the biological activities of the solvent extracts

- Seasonal variation is an important factor that has an influence on essential oil yield. In addition, the chemical profile of the solvent extracts and essential oils were found to vary between seasons.
- It is difficult to determine a specific time of the year when the solvent extract and essential oil provide optimal biological activity as the activity varies with the species involved, as well as the biological activity. However, it was noted that, collection and preparationsof the solvent extracts in winter and essential oils in spring were generally less toxic than plants collected during other seasons.
- The presence of essential oils in the solvent extract had a variable effect on the the activity of the solvent extract and was dependent on the biological activity investigated.
- The solvent extracts containing essential oils were more toxic to human kidney epithelial cells than the solvent extracts devoid of essential oils.
- The results of the *in vitro* pharmacological activities justify why *Salvia* species in general are considered as a 'panacea' in traditional medicine.

14.2 Recommendations

- The collection of the same species from different localities is important in order to determine chemotypic variation for each species.
- The leaf trichome types, biological activity and phytochemistry of indigenous *Salvia* species not included in this study should be completed.
- Investigate the mechanisms by which the solvent extracts and essential oils exert their anti-inflammatory and anti-oxidant activities.
- Isolate the active compounds from the most promising plant which displayed anticancer, antimycobacterial and anti-oxidant activity.
- Further research should consider testing various fractions of the solvent extracts for different biological activities and disregard the fraction with highly toxic effects.
- Perform *in vivo* tests with those plants that exhibited specific activity *in vitro*.

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Appendix A: Abstracts of papers published/submitted from this thesis

Appendix B: Abstracts of conferences/presentations

Appendix B1: (South African Association of Botanists, Durban 2004)

Biological activities and essential oil composition of *Salvia repens*, *S. runcinata* and *S. stenophylla*

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Salvia species have been used medicinally throughout the word. There are about 900 *Salvia* species of which 26 are found in South Africa. The methanol extracts and essential oils from *S. stenophylla*, *S. repens* and *S. runcinata* were investigated for anti-oxidant (α,α-diphenyl-β-picrylhydrazyl), anti-inflammatory (5-lipoxygenase), antimalarial (tritiated hypoxanthine incorporation), antimicrobial (disc diffusion and microplate methods) and toxicity properties (MTT assay). The results indicated that some extracts and/or essential oils were active with the anti-oxidant IC_{50} values ranging from 8.98 to 15.07 ppm (methanol extracts) and anti-inflammatory activity from 22.84 to 30.24 ppm (essential oils). Essential oils and extracts of plants possessed antimalarial properties but are potentially toxic. *S. stenophylla* exhibited the highest activity with the MIC values ranging from 0.10 mg/ml (*S. aureus*) to 3.13 mg/ml (*S. epidermidis*). Essential oils have shown little antimicrobial activity against all tested organisms. Analytical techniques (GC-MS, HPLC) were employed to complete chromatographic profiles for the essential oils and methanol extracts, respectively. HPLC results indicate the presence of rosmarinic acid and carnosic acid derivatives. Using bioassay guided fractionation the antimicrobial activity is ascribed to carnosic acid. The results of the study gave the evidence of the traditional use of the members of the *S. stenophylla* species complex to treat different ailments in some regions of South Africa.

Appendix B2 (Indigenous Plant Use Forum, Grahamstown 2005)

Biological activities of solvent extracts and essential oils of four related *Salvia* species

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Salvia species (sage) are widely used in traditional medicine throughout the world to treat various ailments. In South Africa, they have been used to treat microbial infections, malaria, sore throat and cancer. The solvent extracts and essential oils of four related species; *S. africana-lutea*, *S. africana-caerulea*, *S. lanceolata* and *S. chamelaeagnea* were investigated for antibacterial, antimalarial, anti-inflammatory, anticancer and anti-oxidant activities. The essential oils were obtained by hydrodistillation of the fresh aerial plant parts. The solvent extract (methanol:chloroform) displayed antibacterial activity against Gram-positive and Gram-negative bacteria with MIC values ranging from 0.03 to 4.67 mg/ml. The extracts also exhibited good anti-oxidant ($12.75 \pm 1.04 < IC_{50} < 68.09 \pm 3.69$ µg/ml), antimalarial ($15.86 \pm 5.04 < IC_{50} < 28.68 \pm 2.80$ µg/ml) and anticancer activities ($8.72 \pm 1.52 < IC_{50} < 54.40 \pm 4.42$ µg/ml) but poor anti-inflammatory activity ($IC_{50} > 100$ µg/ml). The essential oils exhibited better antimalarial activity ($4.76 \pm 0.48 < IC_{50} < 8.63 \pm 0.95$ µg/ml) and anti-inflammatory activity ($43.38 \pm 0.48 < IC_{50} < 77.2 \pm 6.27$ µg/ml), antibacterial and poor anti-oxidant activity. The *in vitro* assessment of the toxicity against human kidney epithelium cells indicated that both essential oils and solvent extracts displayed some degree of toxicity with essential oils being more toxic than the solvent extracts. However, the toxicity of the solvent extract was reduced when tested after extraction of the essential oil. A study of *S. chamelaeagnea* and *Leonotis leonurus* in combination showed that when the two plants are used in combination (as reported in the

ethnobotanical literature) they have synergistic effects against Gram-positive bacteria and additive or antagonistic effects against Gram-negative bacteria. The bio-autographic-guided fractionation method was employed to isolate the active compound from *S. chamelaeagnea* against *Staphylococcus aureus*; and was identified by NMR to be carnosol.

Appendix B3 (University of Johannesburg Symposium, 2005)

Trichome morphology and the role of leaf essential oils in the pharmacological activity recorded for indigenous *Salvia* species

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South Africa is home to 26 *Salvia* species of which many are used in traditional medicine to treat various ailments. We investigated the leaf trichome morphology of four related species; *S africana-caerulea, S. africana-lutea*, *S. chamelaeagnea* and *S. lanceolata*. The SEM and the LM show both glandular and non-glandular trichomes. Glandular trichomes are single- or multi-celled with short to medium stalk (1-3 cells) and present in all four species. The non-glandular trichomes are unbranched with at least two stalk cells. The leaf trichome morphology cannot be used to set up boundaries among the four species studied. The present work also investigated the *in vitro* biological activities of extracts in the presence and absence of essential oil to establish the role of the essential oil fraction in the pharmacological activity. The MIC values for the antibacterial activity were best after removal of essential oils against Gram-positive bacteria while the opposite results were obtained for the Gram-negative bacteria. Superior anti-oxidant activity (lower IC_{50} values) and lower toxicity (high IC_{50} values) were obtained after the essential oils were removed from the plants. The total phenolic content was also high after removal of the essential oil fraction. The results suggest that essential oils may play an important role (antagonistic or synergistic) in the biological activity of medicinal aromatic plants. This work forms part of a greater project to summarise the chemistry, trichome morphology and biological activity of indigenous aromatic plants in monograph format.

Appendix B4 (South African Association of Botanists, Port Elizabeth, 2006)

Biological activities, essential oils composition and leaf trichome morphology of four selected *Salvia* species indigenous to southern Africa

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Salvia species are widely used in traditional medicine to treat various ailments. Twenty-three species are indigenous to southern Africa and have been poorly investigated. The biological activities of the essential oils and the solvent extracts of four related species namely *S. africaca-caerulea*, *S. africana-lutea*, *S. chamelaeagnea* and *S. lanceolata* were studied. The essential oils were extracted by hydrodistillation and analyzed by GC and GC/MS. The essential oils displayed good antimalarial (IC_{50} < 8. 6 µg/ml) and promising anti-inflammatory properties (IC_{50} < 77 µg/ml) in comparison to respective controls. The solvent extracts also exhibited potent antimalarial (IC_{50} < 26 µg/ml), anticancer (IC_{50} < 50 µg/ml), antituberculosis (MIC ≤ 0.5 mg/ml) activities but poor anti-inflammatory properties (IC_{50} > 100 µg/ml). The toxicity of the essential oils was higher compared to that of solvent extracts ($P < 0.05$) with the IC_{50} values ranging from 1.87 to 7.24 µg/ml and from 14.38 to 25.01 µg/ml respectively. The analysis of the leaf trichome morphology with SEM and LM revealed the presence of the non-glandular and glandular trichomes in all four species. The non-glandular trichome, unbranched, composed of 2 to 3 stalk cells

was common to all the species investigated. The glandular trichomes, were single or multicelled in the head, 2-4 short/medium stalk cells were also common to the four species. The composition of the essential oils analysis is also reported and the taxonomical relationship using the essential oils data discussed.

Appendix B5 (4th International Conference on Pharmaceutical and Pharmacological Sciences, Gauteng, 2006)

The Antimalarial Activity and Cytotoxic Effects of Solvent Extracts of South African *Salvia* Species and Isolated Compounds from *S. radula*

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Purpose:

About 80% of the population in developing countries, including South Africa rely on traditional medicine for their primary care needs. The objectives of this paper are to investigate the *in vitro* antimalarial and cytotoxic effects of *Salvia* species which are currently used in traditional medicine in South Africa and to isolate the compounds responsible for the antimalarial activity from the most active species.

Method:

The hypoxanthine radioisotope and the sulforhodamine B methods used to investigate the antimalarial and anticancer activity respectively. The cytotoxicity of the extracts (methanol:chloroform) were tested on a human kidney epithelium cell line using the 3-(4,5-dimethylthiazol-2-yl)-2,5 dimethyl tetrazolium bromide (MTT) method and on three human cancer cell lines (SF-268, MCF-7 and HT-29). The active compounds were isolated using a combination of TLC and column chromatography and their characterization was done using NMR and mass spectrometry.

Results:

Solvent extracts displayed antimalarial activity with the IC_{50} values ranging from 3.91 ± 0.52 to 26.01 ± 2.95 µg/ml. Among the plants screened, *S. radula* displayed the most favorable activity, while *S. lanceolata* was the least active. Since the most active

extract was that obtained from *S. radula*, this species was selected to isolate the compound(s) which may account for the observed biological activity. Two compounds were isolated, identified and characterized as; 5-hydroxy-6,7-dimethoxy-2-(4-methoxyphenyl)-4H-1-benzopyran-4-one and betulafolientriol oxide and tested for antimalarial activity. The plant extracts also exhibited the ability to inhibit cell proliferation against the human kidney epithelial cells (12.12 ± 2.02 < IC_{50} < 53.34 ± 3.90 µg/ml) and three human cancer cell lines. The concentration required to inhibit 50% of cell growth (IC_{50} values) ranged between 9.69 ± 0.92 and 43.65 ± 8.38 and between 8.72 ± 1.52 and 54.40 ± 4.20 µg/ml against the MCF-7 and SF-268 cell lines, respectively. Against the HT-29 cell line, the IC_{50} values ranged from 17.05 ± 3.50 to 57.00 ± 11.67 µg/ml; with *S. lanceolata* being the most active plant. *S. africana-caerulea* was the most active against SF-268 and *S. radula* showed the best activity against the MCF-7 cell line. Results obtained in this *in vitro* study may support the traditional use of indigenous *Salvia* species as coveted ingredient in traditional headlining.

Appendix C: Monographs of *Salvia* species investigated

A monograph of all the investigated species are presented in alphabetical order and includes:

(i) a brief description,

(ii) the geographical distribution,

(iii) the traditional uses,

(iv) the main components of the essential oil,

(v) the phenolic compounds identified in each solvent extract,

(vi) the biological activities of the essential oils and solvent extracts,

(vii) the leaf trichome types, and

(viii) the HPLC profile of the solvent extract (only peak with percentage area greater than 3% is recorded).

C1. *Salvia africana-caerulea* L.

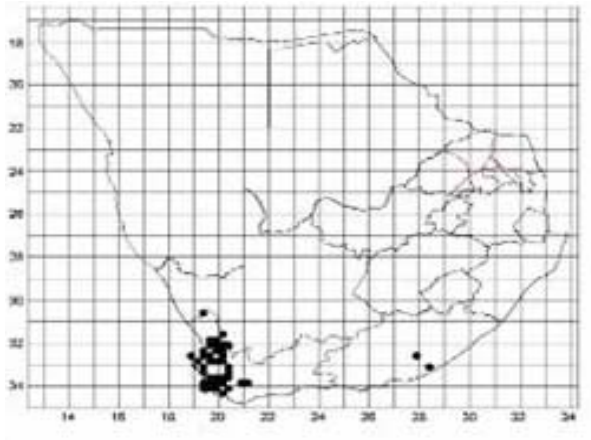
Synonyms: *S. africana* L., *S. lanuginose* Burm.f., *S. integerrima* Mill., *S. barbata* Lm.

Common names: 'Bloublomsalie', wild sage.

Botanical description: Shrub up to 2 m tall, often branching at the base with several erect, usually sparingly branched stems. Stems are gland-dotted and occasionally with glandular hairs. Leaves are petiolated, blade simple, obovate-ellicptic to broadly obovate. The calyx is somewhat funnel-shapped, glandular-villous and the corolla is light blue to bluish purple or pinkish, the lower lip usually with a paler blue margin and white to yellowish in the centre (Codd, 1985).

D**istribution:** This plant is distributed from the Vanrhynsdorp district to Cape Town and eastwards to Montagu and Caledon with an old record from Peddi district in the Eastern Cape Province, common in coastal fynbos and rocky slopes (Codd, 1985).

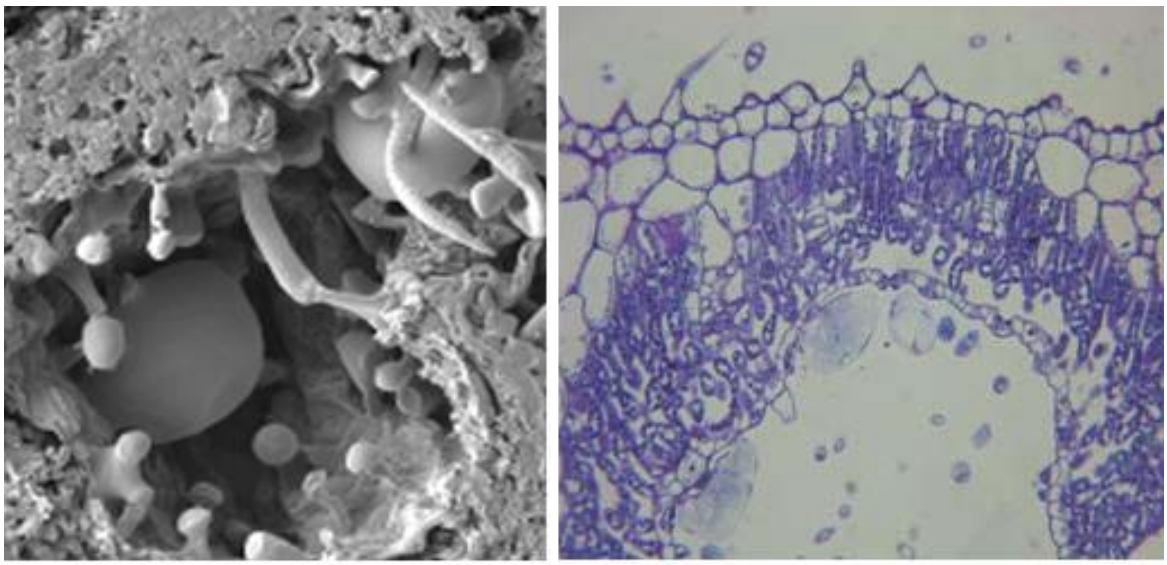
Petiolate and obovate leaves and flowers of *S. africana-caerulea.*



Distribution of *S. africana-caerulea.*

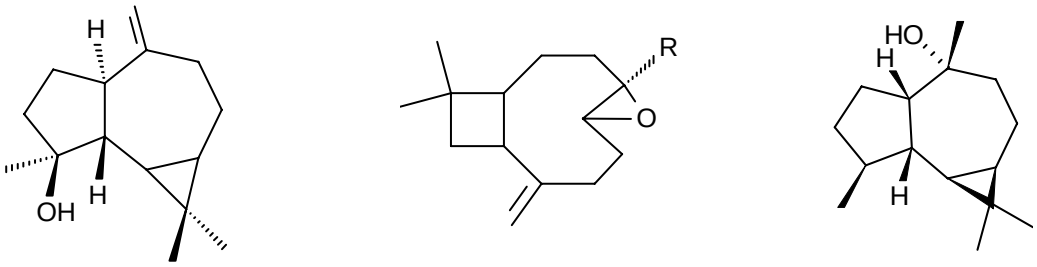
Traditional uses: The infusion or decoction is used as a remedy for coughs, colds, chest problems, urine troubles, kidney infections, stomach ailments (Watt and Breyer-Brandwijk 1962; Hutchings *et al*.,1996; van Wyk *et al*. (1997).

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



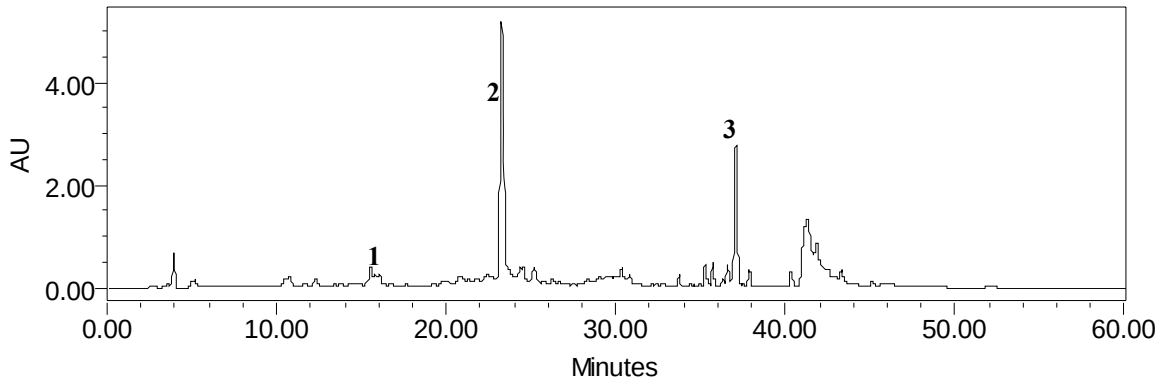
Scanning electron microscopy and light microscopy micrographs of *S. africana-caerulea.*

Essential oil composition: The essential oil yield of this plant collected in the South Western Cape was 0.12% (w/w, wet biomass) and the major components of this oil include spathulenol (29.1%), β-caryophyllene oxide (14.6%) and ledol (6.5%).



Spathulenol β-Caryophyllene oxide Ledol

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, 7-*O*-methylepirosmanol, salvigenin, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were detected. The HPLC chromatogram and the tentative of identification are displayed in below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. africana-caerulea*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. africana-caerulea*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	15.55	218, 324	Caffeic acid	5.29
2	23.27	329	Rosmarinic acid	22.90
3	37.08	204, 283		11.55

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	8.72	23.36	27.10	> 100.0	22.68	39.72	33.40	14.38	115.06
EO	nd	nd	nd	73.47	4.76	> 100.0	> 100.0	1.87	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are displayed below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	4.67	6.00	3.00	0.50
EO	16.00	8.00	0.75	4.00	nd

nd: not determined

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Watt, J.M., Breyer-Brandwijk, B.N. 1962. *Medicinal and Poisonous Plants of Southern and Eastern Africa*. 2nd edition. E. and S. Livingstone, Edinburg, UK.

C2. *Salvia africana-lutea* L.

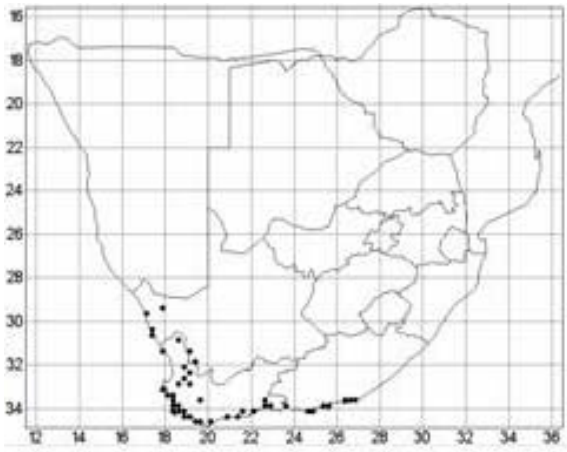
Synonyms: *S. aurea* L., *S. colorata* L., *S. eckloniana* Benth.

Common names: 'Bruinsalie', 'beach Salvia', 'Dune Salvia', 'golden Salvia'

Botanical description: Branched shrub up to 2 m tall; stems are densely leafy. Leaves are petiolate; blade simple, grey-tomentosed, gland dotted, apex rounded to obtuse. The calyx is purplish and membranous with short spreading glandular and non-glandular hairs dotted with orange-red gland-dots. The corolla is golden brown, reddish brown khaki or sometimes purplish. The flowering begins in early spring and the bright yellow flowers soon fade to rusty-orange and then reddish brown (Codd, 1985).

Distribution: The distribution of *S. africana-lutea* extends from the coast of Namaqualand to the Cape Peninsula and eastwards from there to Port Alfred. In its natural state, it grows not far from the sea and is often a common constituent of the vegetation on coastal sand dunes (Codd, 1985).

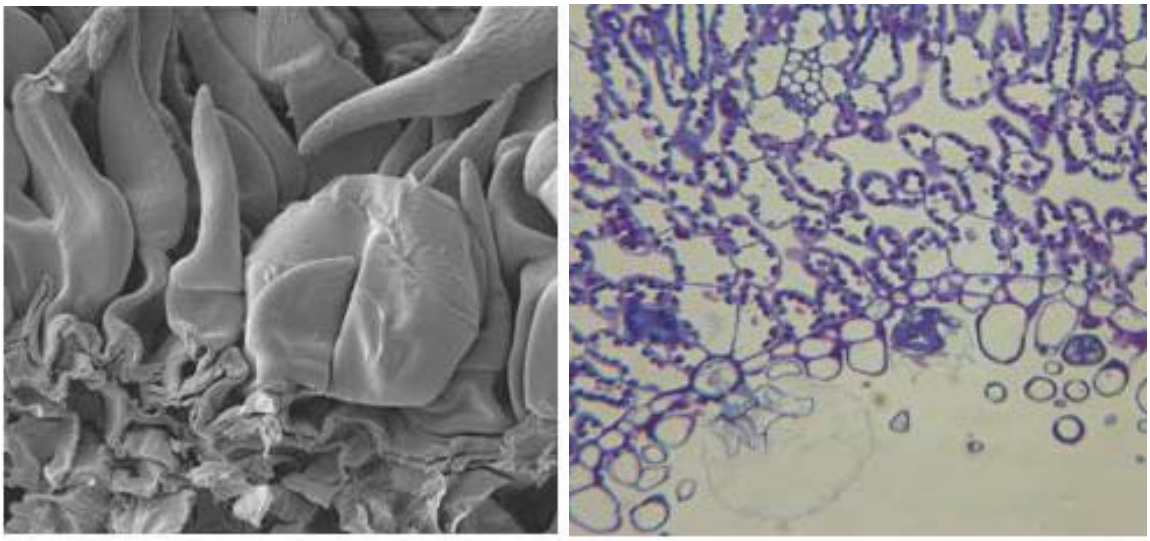
Golden brown flowers of S. *africana-lutea.*



Distribution of S. *africana-lutea.*

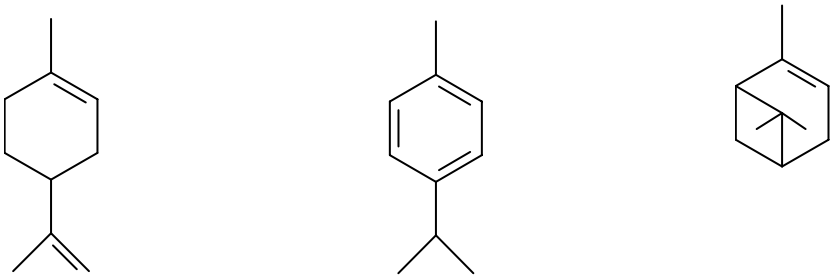
Traditional uses: An infusion of the plant is used for coughs, colds and female ailments (Watt and Breyer-Brandwijk, 1962).

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



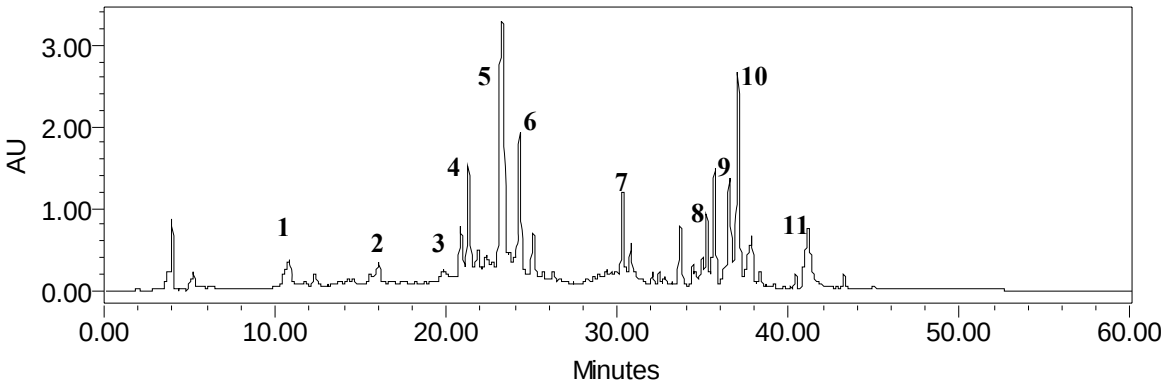
Scanning electron microscopy and light microscopy micrographs of *S. africana-lutea*.

Essential oil composition: The essential oil yield of this plant collected in the South Western Cape was 0.15% (w/w, wet biomass) and the major components in the oil include myrcene (11.5%), *p*-cymene (7.6%) and α-pinene (6.0%).



Myrcene *p*-Cymene α-Pinene

HPLC analysis of the solvent extract: Rosmarinic acid, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. africana-lutea*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. africana-lutea*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	10.81	281, 374		3.47
2	16.03	202, 281, 391		3.12
3	19.79	273		3.51
4	21.30	255, 283, 345		8.58
5	23.21	311, 326	Rosmarinic acid	23.82
6	24.28	286, 310		3.10
7	30.31	202, 285, 328		9.20
8	35.68	205, 282		7.46
9	36.53	284, 391		3.2
10	37.05	208, 283	Carnosic acid	14.22
11	41.17	245, 277, 374		4.49

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in the table below.

	AC			**AI**	**AM**	**AO**		**TO**	**TP**
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	43.65	20.00	> 100.0	15.86	30.35	47.58	25.01	67.83
EO	nd	nd	nd	77.32	5.45	> 100.0	> 100.0	7.24	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are displayed in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	3.00	3.00	0.75	0.75	0.50
EO	> 32.00	8.00	2.33	8.00	nd

nd: not determined

References

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

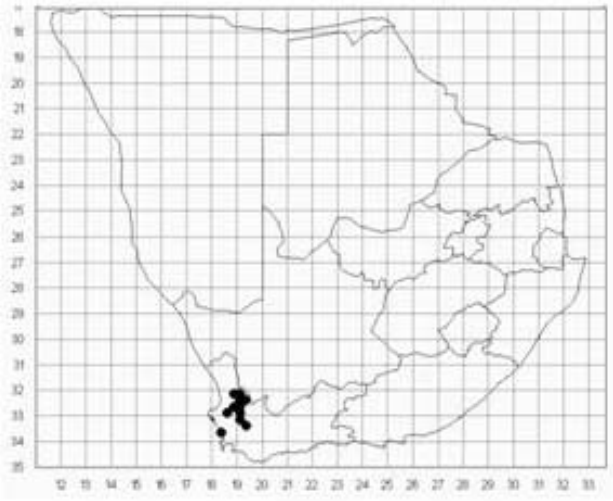
Watt, J.M., Breyer-Brandwijk, B.N. 1962. *Medicinal and Poisonous Plants of Southern and Eastern Africa*. 2nd edition. E. and S. Livingstone, Edinburg, UK.

C3. *Salvia albicaulis* Benth.

Synonym: *S. dregeana* Benth.

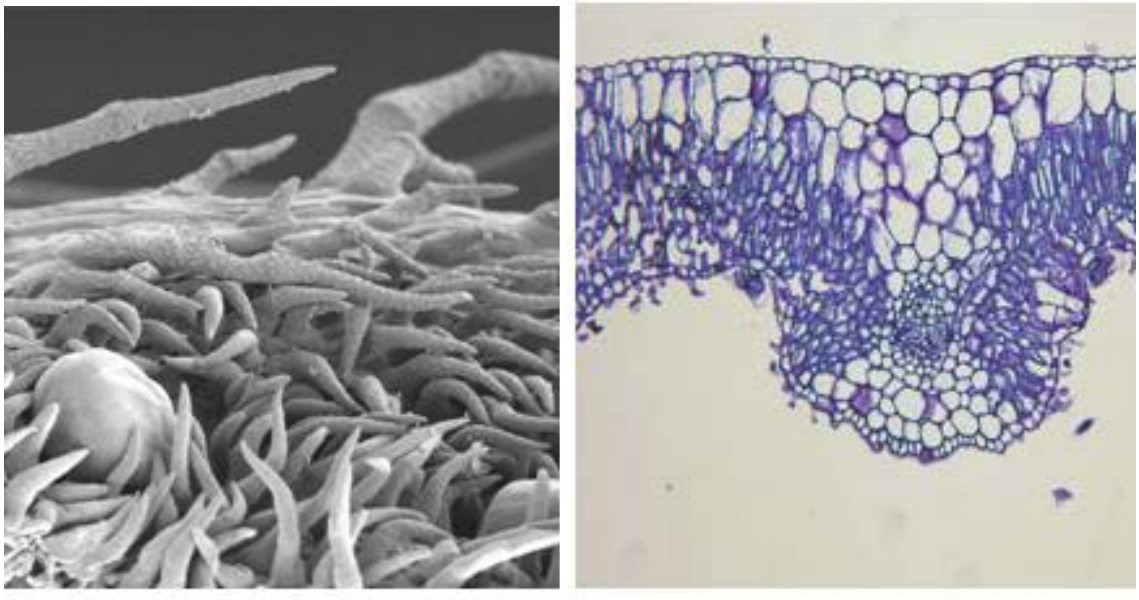
Botanical description: Shrub or woody herb up to 0.3-0.6 m tall and branched from the base. Stems erect, densely and shortly tomentose with occasional multicelular hairs. Leaves are petiolated, blade simple and coriaceous. The inflorescence is a panicle. Calyx is densely villous with long white hairs mainly along the nerves and the corolla is purplish (Codd, 1985). Flowering time is November to May.

Distribution: This species is distributed from Clanwilliam southwards to Ceres and Wellington districts, common in fynbos on rocky slopes (Codd, 1985).



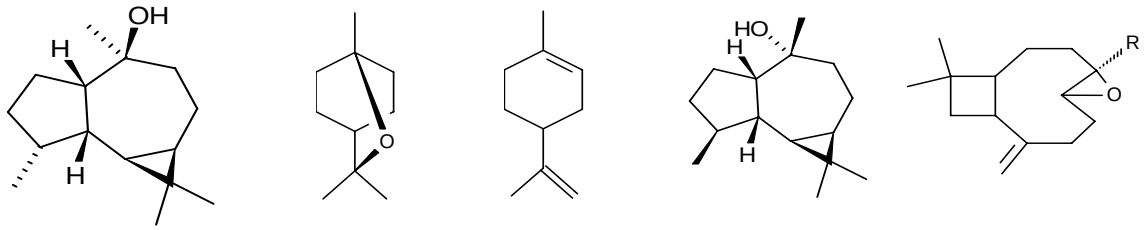
Distribution of *S. albicaulis*

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



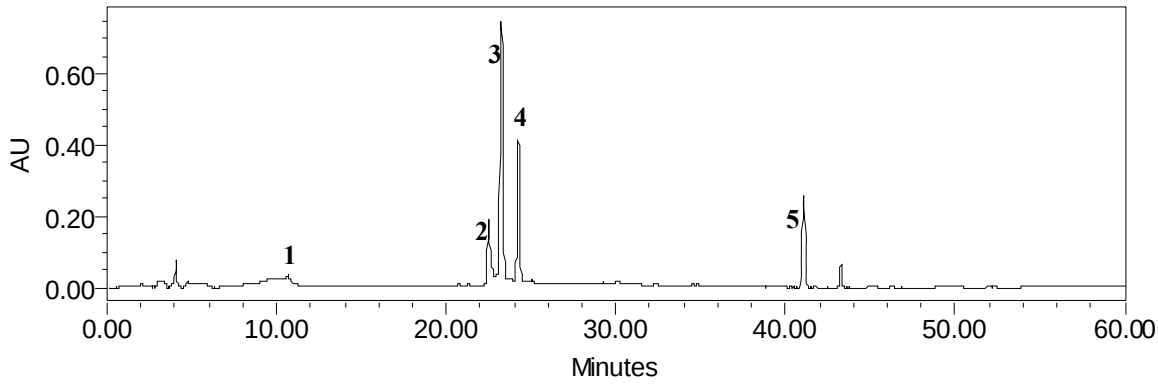
Scanning electron microscopy and light microscopy micrographs of *S. albicaulis*.

Essential oil composition: The essential oil yield of this plant collected at the Kirstenbosch Botanical Garden was 0.06% (w/w, wet biomass) and the major components include viridiflorol (24.5%), 1,8-cineole (9.4%), limonene (9.4%), ledol (6.6%) and β-caryophyllene oxide (5.6%).



Viridiflorol 1,8-Cineole Limonene Ledol β-Caryophyllene oxide

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, salvigenin, carnosol, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table which follows.



HPLC chromatogram of the solvent extract of *S. albicaulis*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. albicaulis*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	10.67	282, 327, 357		9.54
2	22.48	228, 284, 327		6.36
3	23.24	330	Rosmarinic acid	35.23
4	24.25	230, 256, 286		14.58
5	41.08	373		10.23

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in the table, which follows.

	AC			**AI**	**AM**	**AO**		**TO**	**TP**
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	27.50	35.25	25.33	> 100.0	15.83	24.22	19.85	37.29	100.27
EO	nd	nd	nd	39.22	6.41	> 100.0	> 100.0	2.75	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method, nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are depicted in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	4.67	1.00	1.00	0.50
EO	> 32.00	8.00	2.33	8.00	nd

nd: not determined

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

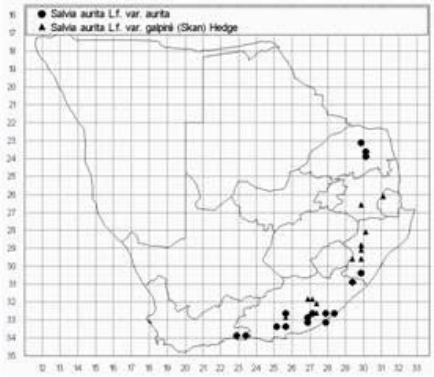
C4. *Salvia aurita* L. f.

Synonyms: *S. sylvicola* Burch., *S. lasiostachys* Benth., *S. peglerae* Skan

Botanical description: Perennial herb with few to several stems from a subwoody base, stems ascending or straggling, pilose, usually with longish multicelular hairs, sometimes dense, or short and dense and occasionally with capitate glandular hairs. Leaves are petiolated, blade simple to lyrate or runcinate and variable in shape. Apex obtuse to rounded, base truncate to auriculate. Calyx pilose, tubular campanulate, corolla pale blue, lilac or white pinkish (Codd, 1985).

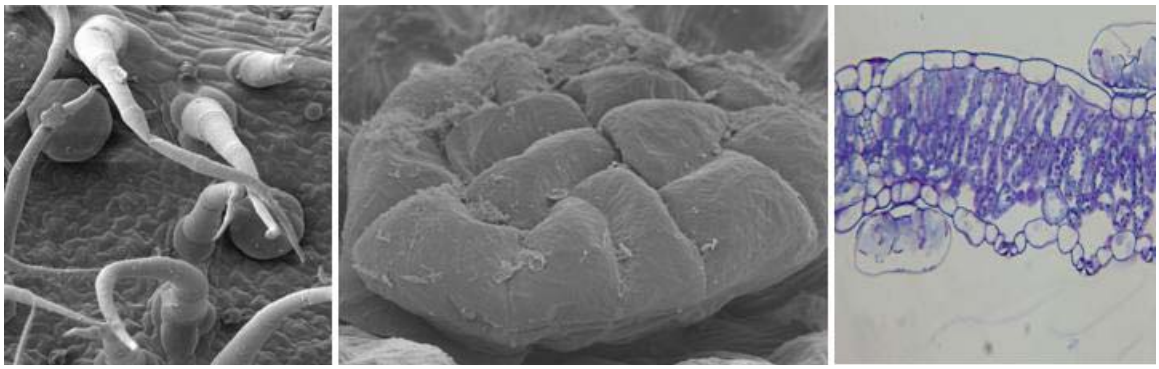
Distribution: This plant is distributed from the southern and south Eastern and KwaZulu-Natal to the Southpansberg in Gauteng, on grassy slopes, stream banks and wooded places (Codd, 1985).

Pilose stem and petiolate leaves of *S. aurita.*



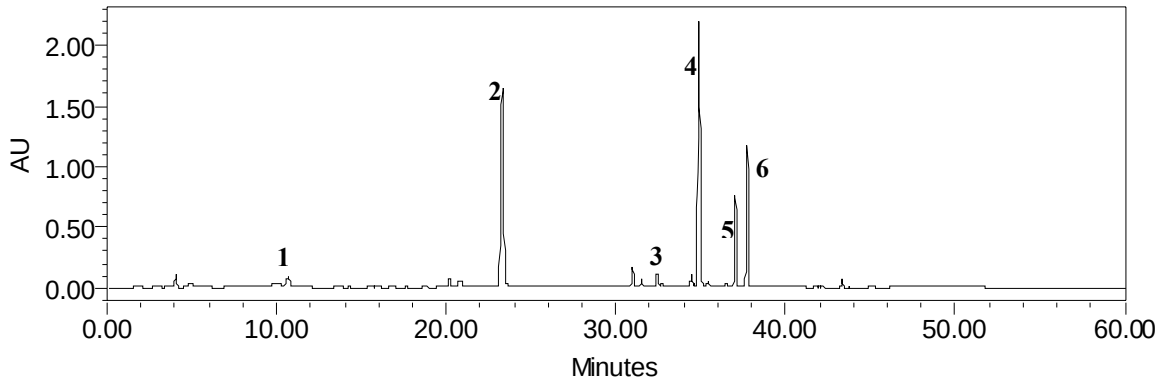
Distribution of *S. aurita.*

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



Scanning electron microscopy and light microscopy micrographs of *S. aurita.*

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are given in the table.



HPLC chromatogram of the solvent extract of *S. aurita*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. aurita*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	10.67	282, 341, 355		5.63
2	23.29	330	Rosmarinic acid	24.44
3	32.44	235, 285, 333		3.06
4	34.90	206, 284, 319	Carnosol	27.61
5	37.05	285	Carnosic acid	6.37
6	37.74	205, 283		20.74

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the total phenolic content are given in table, which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	44.87	17.28	24.58	> 100.0	8.92	22.94	16.59	28.31	119.09

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract against various bacteria are given in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	4.00	0.20	0.05	0.50

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

C5. *Salvia chamelaeagnea* Berg.

Synonym: *S. paniculata* L.

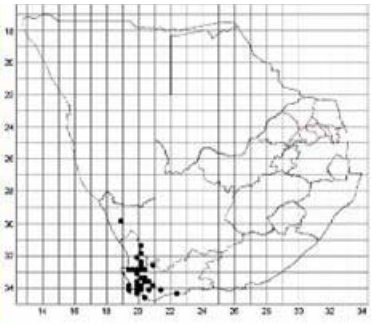
Common name: 'Afrikaansesalie'

Botanical description: *Salvia chamelaeagnea* forms a dense shrub, growing almost 2 meters high. The stems are square-shaped, a typical feature of the Lamiaceae. The leaves are dotted with glands which emit a very strong scent. The large calyx is usually an attractive reddish-purple colour. The corolla usually called the flower varies in colour from blue, mauve, pink to pure white. Flowers with a dark blue top lip and white lower lip are the most common (Codd, 1985). Flowering time is from October to May.

Distribution: This plant is distributed from Clanwilliam to Cape Town and eastwards to Ladismith and Riversdale districts, but also found in fynbos along watercourses, in sandy soil among rocks and along roadsides (Codd, 1985).

Petiolate and elliptical leaves of *S. chamelaeagnea.*

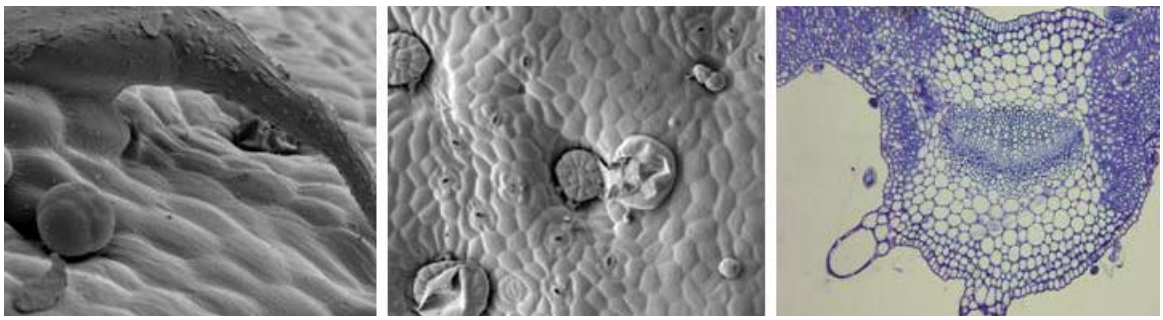
Large panicle inflorescence of *S. chamelaeagnea.*



Distribution of *S. chamelaeagnea.*

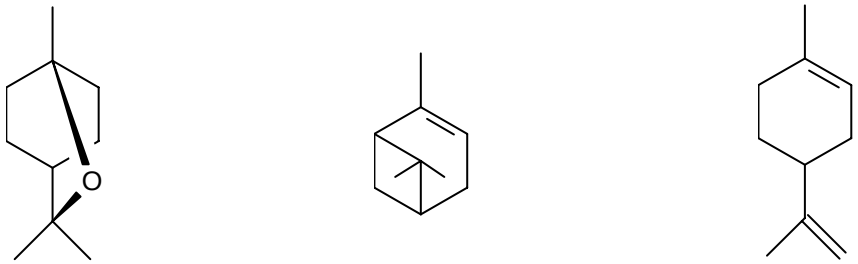
Traditional uses: Like many of the other sages, *Salvia chamelaeagne*a is used to treat a wide variety of ailments such as coughs, symptoms associated with cancer, colds, diarrhoea, colic, heartburn and flatulence. A tea, to be sipped slowly a few times a day, is usually made from the fresh or dried leaves and flowers (Watt and Breyer-Brandwijk, 1962).

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



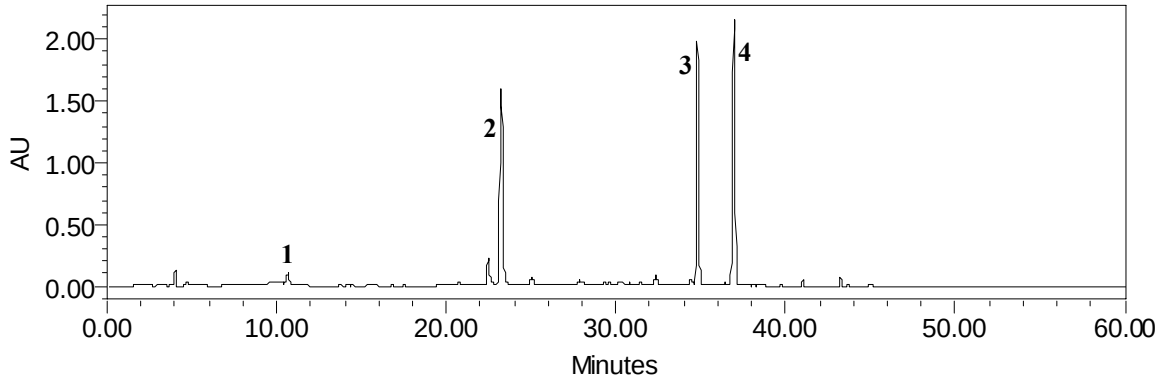
Scanning electron microscopy and light microscopy micrographs of *S. chamelaeagnea*.

Essential oil composition: The essential oil yield of the plant collected at the Kirstenbosch Botanical Garden was 0.39% (w/w, wet biomass) and the major components for the oil include 1,8-cineole (40.5%) followed by α-pinene (10.4%) and limonene (9.7%).



1,8-Cineole α-Pinene Limonene

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, oleanolic acid, 7-*O*-methylepirosmanol, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table which follows.



HPLC chromatogram of the solvent extract of *S. chamelaeagnea*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. chamelaeagnea*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	10.63	282, 339, 354		4.71
2	23.23	330	Rosmarinic acid	26.36
3	34.81	206, 284, 324	Carnosol	22.48
4	36.95	203, 285	Carnosic acid	27.25

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in the table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	34.98	18.12	29.53	> 100.0	8.71	14.61	12.75	24.76	211.78
EO	nd	nd	nd	48.58	8.63	> 100.0	> 100.0	6.00	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against various bacteria are depicted in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	1.00	3.00	0.03	0.06	0.50
EO	> 32.00	12.00	2.00	> 32.00	nd

nd: not determined

References

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

Watt, J.M., Breyer-Brandwijk, B.N. 1962. *Medicinal and Poisonous Plants of Southern and Eastern Africa*. 2nd edition. E. and S. Livingstone, Edinburg, UK.

C6. *Salvia disermas* L.

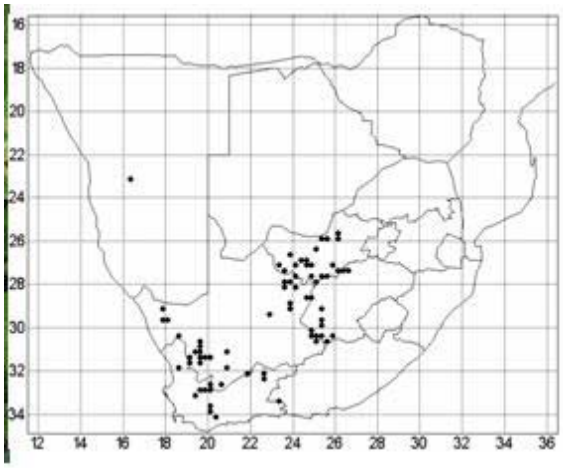
Synonyms: *S. rugosa* Ait., *S. fleckii* Gurke, *S. fleckii* Gürke

Common name: Grootblousalie.

Botanical description: Herbaceous perennial shrub, up to 1.2 m tall with one or more stems from a woody rootstock. Leaves are often crowded and larger near the base of the plant and petiolated. The inflorescence is composed of 15 or more verticils. The calyx is glandular-hispid to villous, while the corolla is whitish, pale blue or mauve (Codd, 1985). The flowering time is from August to May.

Distribution: This plant is distributed in the south-west of Gauteng and Northern Cape through the western Free State and Namaqualand (Codd, 1985)

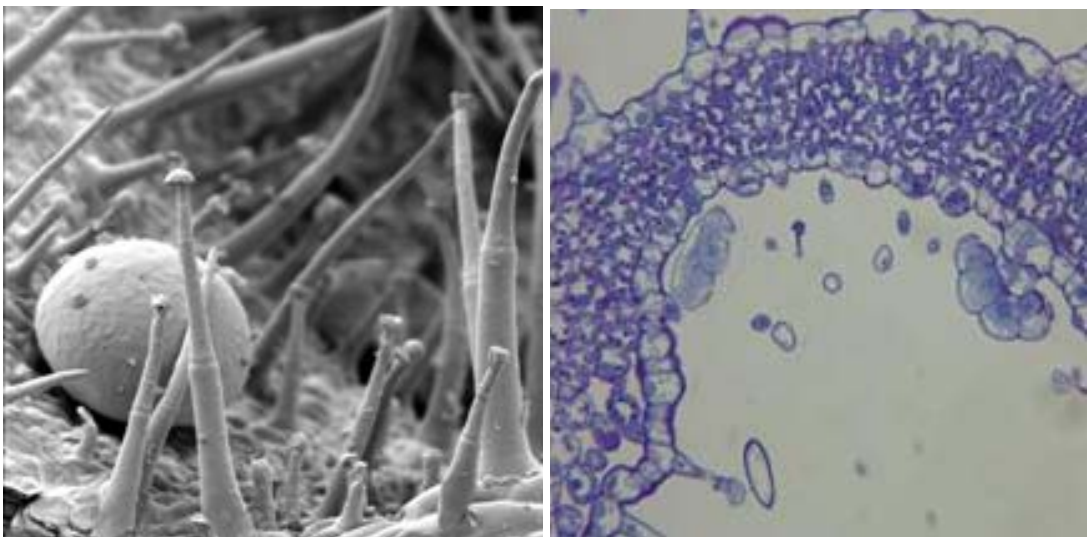
Glandular stems and inflorescence of *S. disermas.*



Distribution of *S. disermas.*

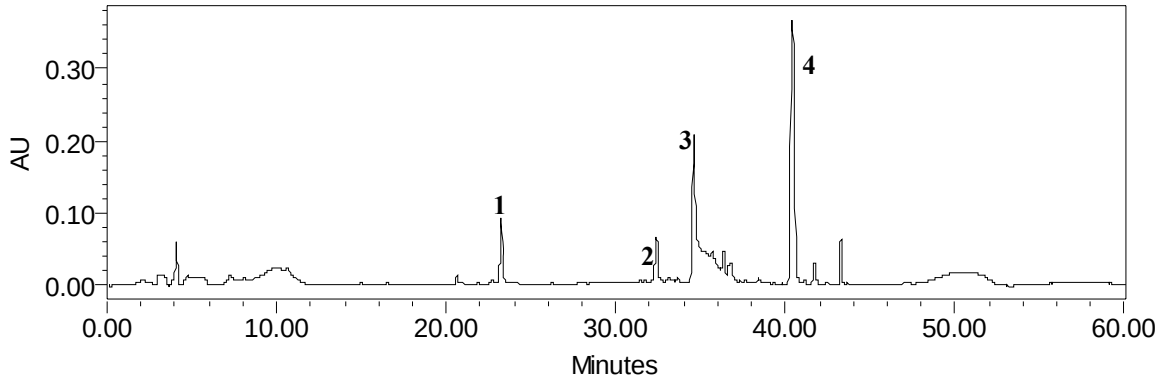
Traditional uses: Treatment of heart problems, high blood pressure, rheumatism and sores; used as a tea (Watt and Breyer-Brandwijk, 1962).

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



Scanning electron microscopy and light microscopy micrographs of *S. disermas*.

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, salvigenin, ursolic acid and betulafolientriol oxide were identified. Various unidentified flavonoids were detected in the solvent extract. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table which follows.



HPLC chromatogram of the solvent extract of *S. disermas.*

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. disermas*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.24	330	Rosmarinic acid	3.62
2	32.40	284, 332		3.35
3	34.58	218, 277, 330	Salvigenin	23.76
4	40.40	271		19.85

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the total phenolic content are given in the table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	59.12	38.56	26.87	> 100.0	24.17	33.05	55.07	53.34	69.01

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) against various bacteria are given in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	2.00	3.00	0.75	0.50

References

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

Watt, J.M., Breyer-Brandwijk, B.N. 1962. *Medicinal and Poisonous Plants of Southern and Eastern Africa*. 2nd edition. E. and S. Livingstone, Edinburg, UK.

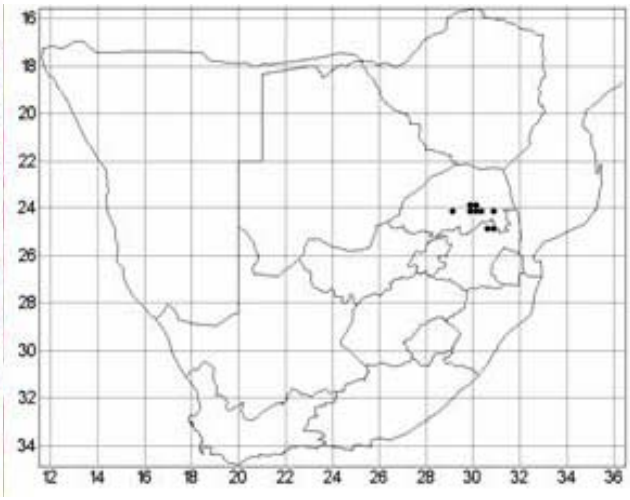
C7. *Salvia dolomitica* Codd

Common name: 'Kruipsalie'

Botanical description: It is a shrub that may reach 2 m in height and branched from the base. Leaves are petiolated, blade simple, elliptic to obovate densely greyish and gland-dotted. The inflorescence is compact. The calyx is broadly campanulate, often purple-tinged, glandular hirsute. The corolla is light pink or lilac with cream or yellow markings on the lower lip (Codd, 1985).

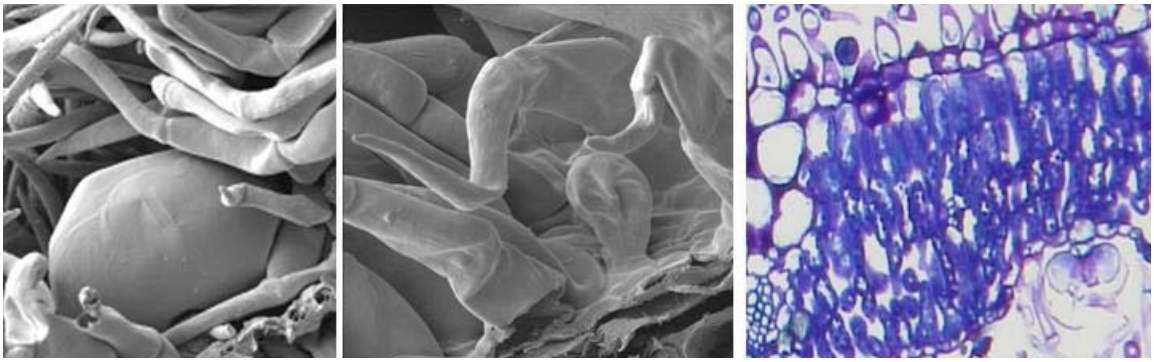
Distribution: The plant is restricted to Gauteng, between 1000 and 1500 m altitude (Codd, 1985).

Leaves and flowers of *S. dolomitica.*



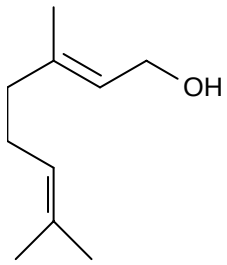
Distribution of *S. dolomitica.*

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.

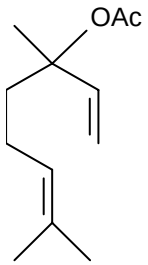


Scanning electron microscopy and light microscopy micrographs of *S. dolomitica*.

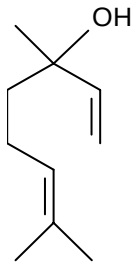
Essential oil composition: The essential oil yield obtained from the plant collected at the Kirstenbosch Botanical Garden was 0.34% (w/w, wet biomass) and the major components for the oil include geraniol (19.6%), linalyl acetate (19.6%), linalool (16.6%) and α-terpineol (6.2%).



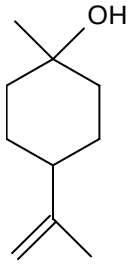
Geraniol



Linalyl acetate

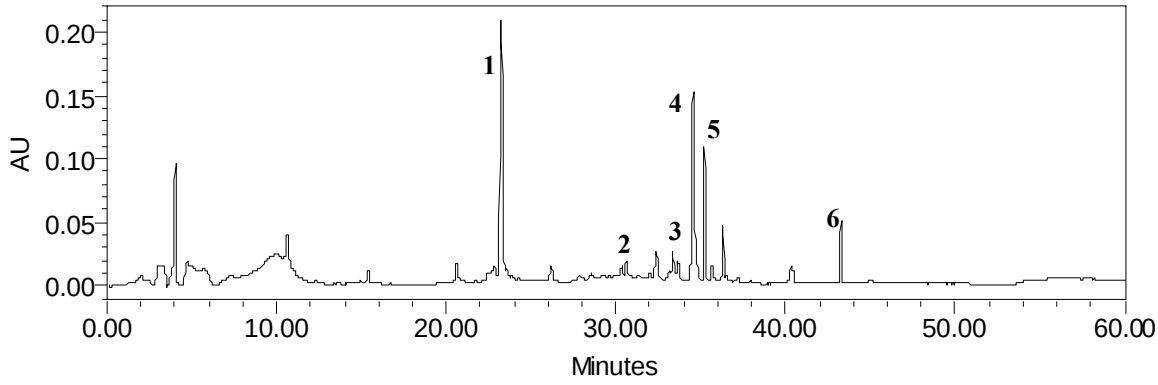


Linalool



α-Terpineol

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table which follows.



HPLC chromatogram of the solvent extract of *S. dolomitica*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. dolomitica*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.23	231	Rosmarinic acid	14.05
2	30.62	252, 268, 341		4.48
3	33.36	252, 269, 344		3.44
4	34.53	237, 276, 329		9.16
5	35.23	242, 326, 373		4.36
6	43.27	249, 273, 355		5.08

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	37.05	> 100.0	> 100.0	7.62	49.94	> 100.0	40.26	53.04
EO	nd	nd	nd	64.58	4.81	> 100.0	> 100.0	7.74	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are given in table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	3.33	2.00	0.75	0.03	0.10
EO	> 32.00	8.00	9.33	12.00	nd

nd: not determined

References

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

Watt, J.M., Breyer-Brandwijk, B.N. 1962. *Medicinal and Poisonous Plants of Southern and Eastern Africa*. 2nd edition. E. and S. Livingstone, Edinburg, UK.

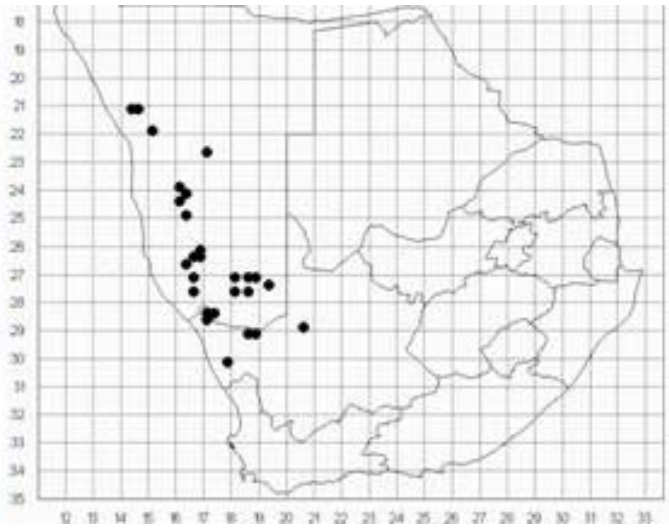
C8. *Salvia garipensis* E. Mey. ex Benth.

Synonyms: *S. steingroeveri* Briq., *S. dinteri* Briq.

Botanical description: Shrub mostly branched up to 1.2 m tall. Stems are glandular-pubescents with longish non-glandular hairs. Leaves are green, glandular-hispid and glandular-dotted. The leaves vary considerably in size, degree of crenation. The calyx is glandular-pubescent and the corolla is white or pale blue to mauve (Codd, 1985).

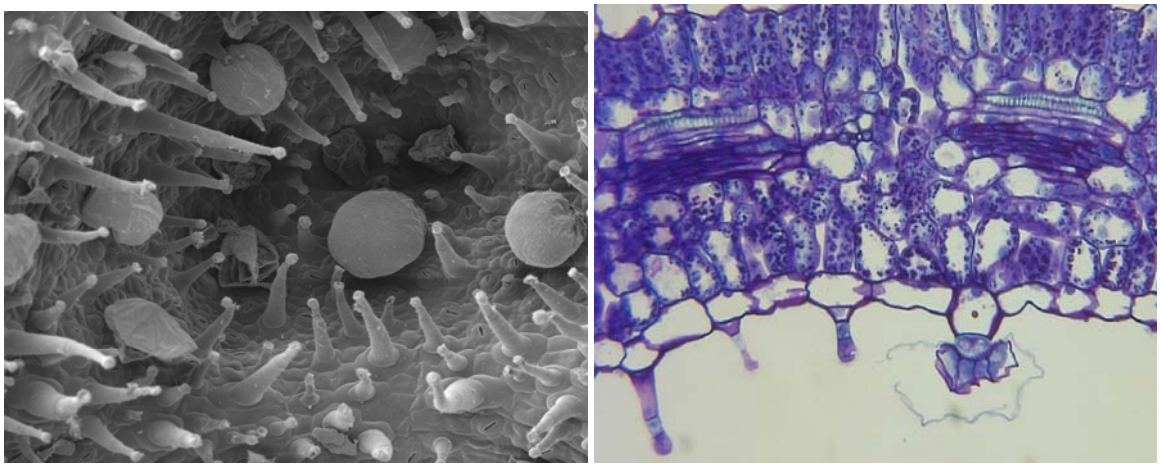
Distribution: This species is distributed from the southern half of Namibia to the adjoining Northern Cape Province (Codd, 1985).

Flowers of *S. garipensis*.



Distribution of *S. garipensis*.

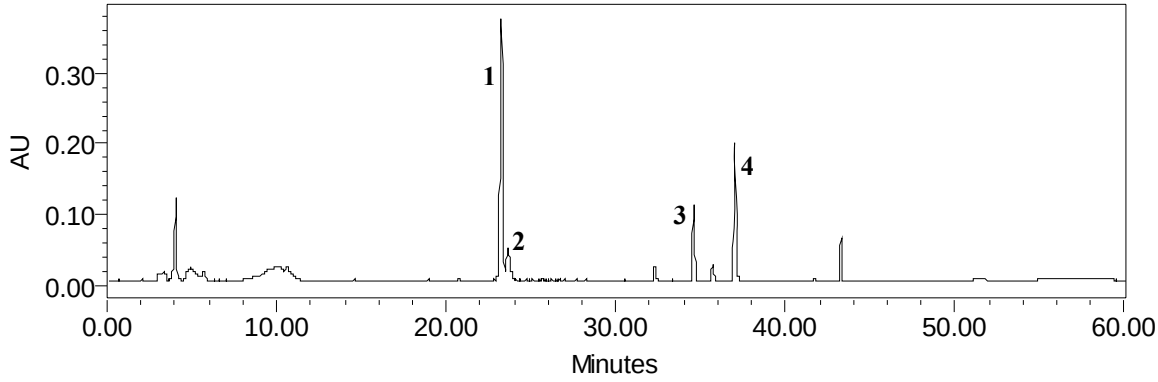
Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



Scanning electron microscopy and light microscopy micrographs of *S. garipensis*.

HPLC analysis of the solvent extract: Rosmarinic acid, salvigenin and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while

the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. garipensis*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. garipensis*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.24	331	Rosmarinic acid	29.22
2	23.63	252, 347	Flavone	4.76
3	34.57	277, 332	Salvigenin	7.38
4	37.02	273, 400		14.09

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the total phenolic content are given in the table which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	ABTS·+	DPPH·	MTT	FCM
SE	> 100.0	39.44	> 100.0	> 100.0	13.95	41.66	74.15	42.44	45.56

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) against bacteria are given in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	1.88	2.81	0.75	4.00	nd

nd: not determined

Reference

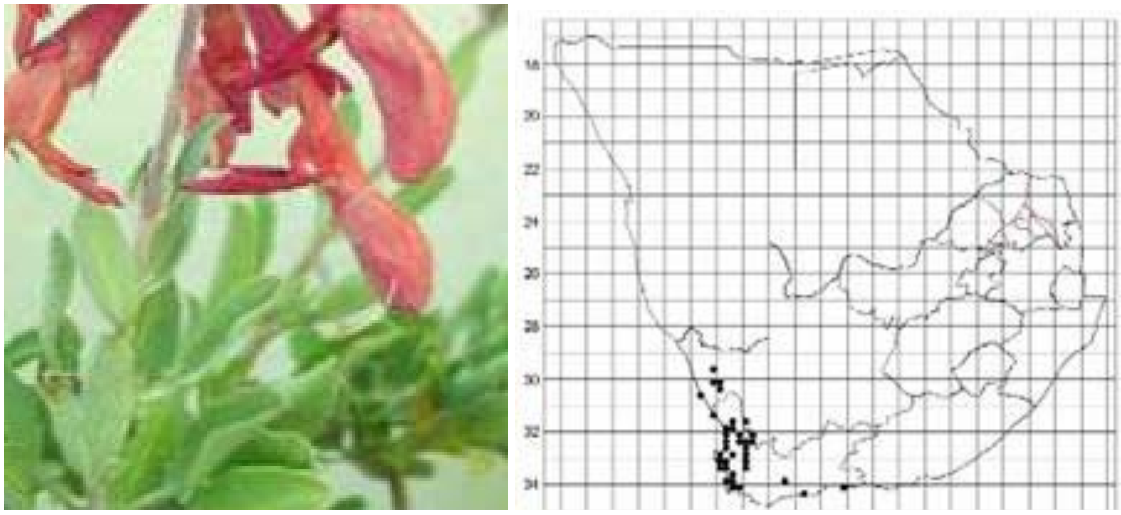
Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

C9. *Salvia lanceolata* Lam.

Synonyms: S. *nivea* Thunb.*, S. hastifolia* Benth., *S. diversifolia* Benth.

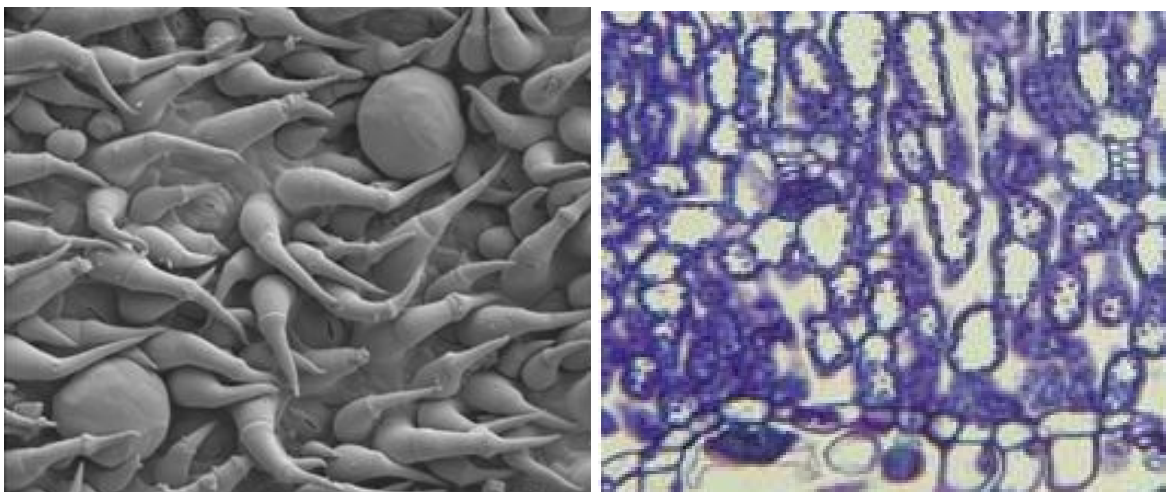
Botanical description: Branched shrub up to 2 m tall, stems glabrescent often reddish brown. Leaves petiolate, blade simple, linear elliptic to ovate-oblong or oblanceolate with a short dense greywish tomentum or with a sparse indumentum of short broad hairs mainly on the veins and leaf margin, gland-doted, apex acute. Inflorescence usually branched. Calyx fairly densely glandular-hispid. Corolla dull rose to brownish crimson or grey-blue (Codd, 1985). Flowering time September to November.

Distribution: This plant is distributed from Namaqualand to the Cape Peninsula and eastwards to Montagu. Found mainly in coastal sandveld or arid fynbos, on sandy soil and rocky hillsides at altitudes of about 300 m (Codd, 1985).



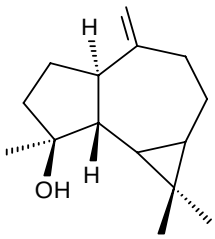
Linear-elliptic leaves, inflorescence and distribution map of *S. lanceolata*

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.

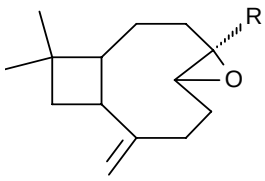


Scanning electron microscopy and light microscopy micrographs of *S. lanceolata*.

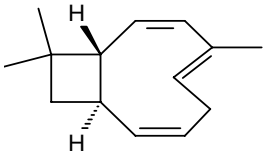
Essential oil composition: The essential oil yield for this plant collected in the South Western Cape was 0.10% (w/w, wet biomass) and the major components include spathulenol (18.3%), β-caryophyllene oxide (14.3%), β-caryophyllene (5.7%) and ledol (5.2%).



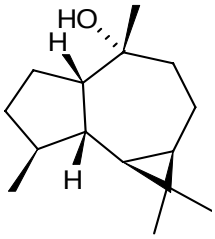
Spathulenol



β-Caryophyllene-oxide

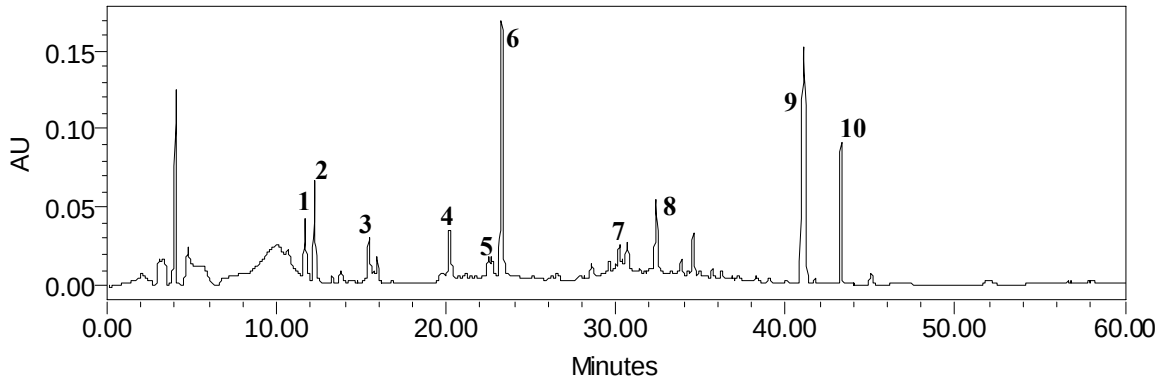


β-Caryophyllene



Ledol

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, carnosol, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. lanceolata.*

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. lanceolata*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	11.66	279, 329, 355		20.58
2	12.22	229, 278, 329		3.35
3	15.44	242, 298, 324	Caffeic acid	3.29
4	20.20	226, 282, 376		3.70
5	22.49	233, 284, 329		3.77
6	23.26	235, 329	Rosmarinic acid	10.67
7	30.66	244, 269, 340		7.57

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
8	32.37	239, 284, 332		6.34
9	41.09	273, 374	Ursolic acid	14.28
10	43.26	248, 373, 374		3.83

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in the table which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	26.15	17.05	> 100.0	26.01	25.63	68.09	26.71	54.22
EO	nd	nd	nd	43.38	7.83	> 100.0	>100.0	3.66	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay ,while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are displayed below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	2.00	3.00	2.00	0.50
EO	32.00	16.00	1.00	8.00	nd

nd: not determined

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

C10. *Salvia muirii* L. Bol.

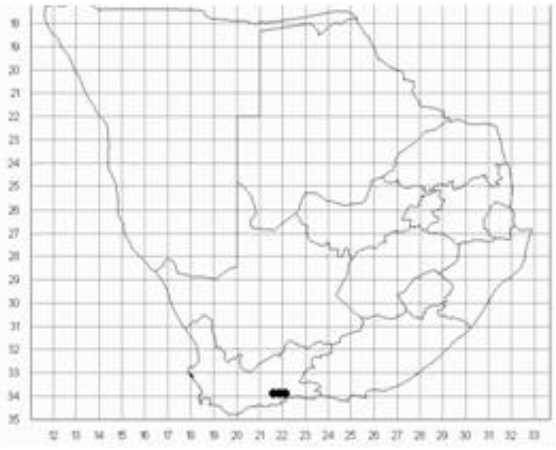
Synonym: *S. muirii var. gradiflora* L. Bol.

Common names: 'Wild sage', 'wildesalie'.

Botanical description: *Salvia muirii* is a small, twiggy, evergreen shrub that grows only about 30 cm high. The leaves are leathery, oval in shape and light green to almost grey in colour. When crushed, they are slightly sticky and release a light scent that is reminiscent of Vicks. The calyx is densely and shortly antrose pubescent, dotted with orange-red glands (Codd, 1985).

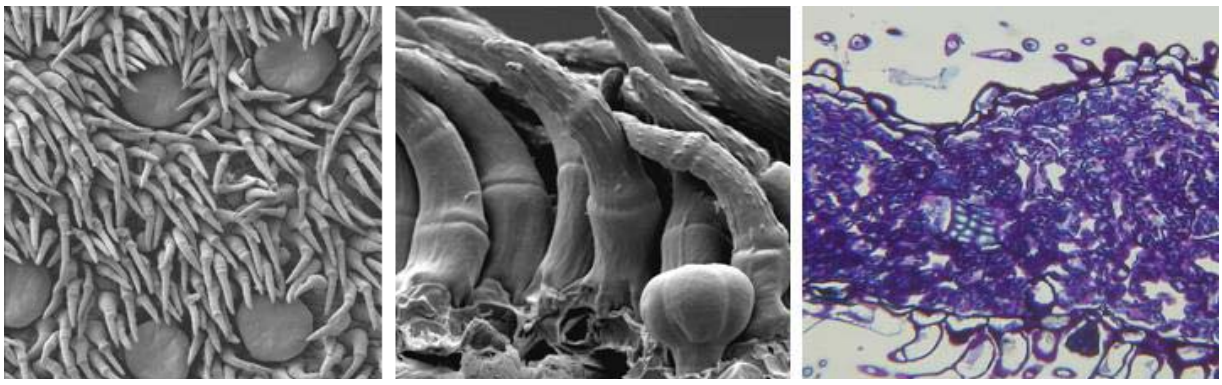
Distribution: This species is found in the Langberg in the Riversdale district to Great Brak River in the Mossel Bay district (Codd, 1985).

Oval leaves and flowers of *S. muirii.*



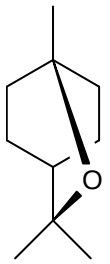
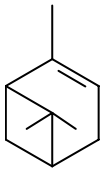
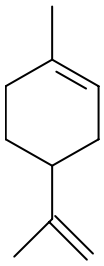
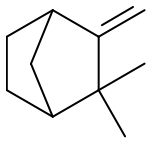
Distribution of *S. muirii*

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



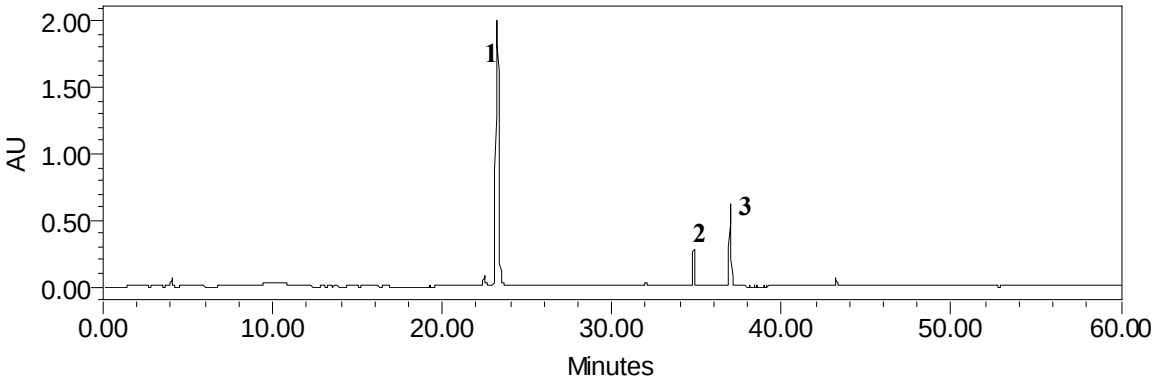
Scanning electron microscopy and light microscopy micrographs of *S. muirii*.

Essential oil composition: The essential oil yield of this plant collected at the Kirstenbosch Botanical Garden was 0.50% (w/w, wet biomass) and the major components include 1,8-cineole (23.2%), α-pinene (22.3%) followed by limonene (11.6%) and camphene (7.6%).

1,8-Cineole α-Pinene Limonene Camphene

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, salvigenin, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table which follows.



HPLC chromatogram of the solvent extract of *S. muirii*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. muirii*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.23	330	Rosmarinic acid	52.82
2	34.82	232, 284, 327	Carnosol	4.83
3	36.98	285, 327, 371	Carnosic acid	11.80

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are displayed in the table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	39.07	55.63	> 100.0	11.87	11.88	11.06	37.00	186.15
EO	nd	nd	nd	> 100.0	5.93	> 100.0	> 100.0	3.09	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are given below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	2.00	3.00	0.25	0.36	0.50
EO	> 32.00	6.00	9.33	> 32.00	nd

nd: not determined

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

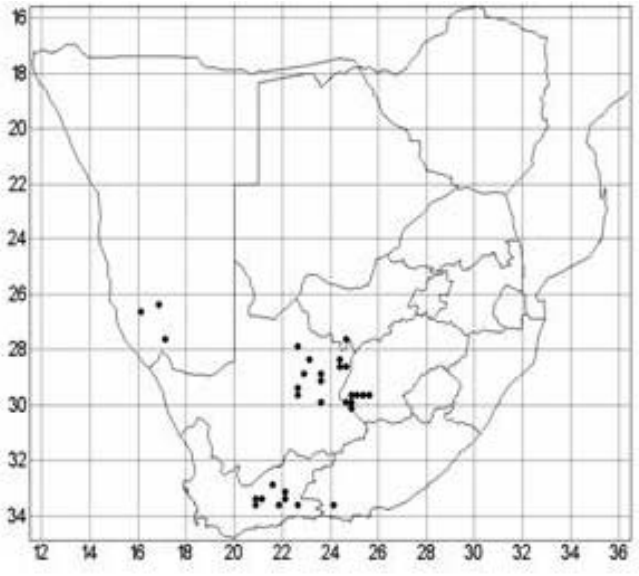
C11. *Salvia namaensis* Schinz

Synonym: *S. burchellii* N.E.Br.

Botanical description: Shrub or bushy shrub up to 1.2 m tall, often herbaceous above and woody below. Leaves are shortly petiolate, blade irregular. The inflorescence is simple, of up to 14 verticils. The calyx and glandular-hispidulous and the corolla is white, mauve or blue (Codd, 1985).

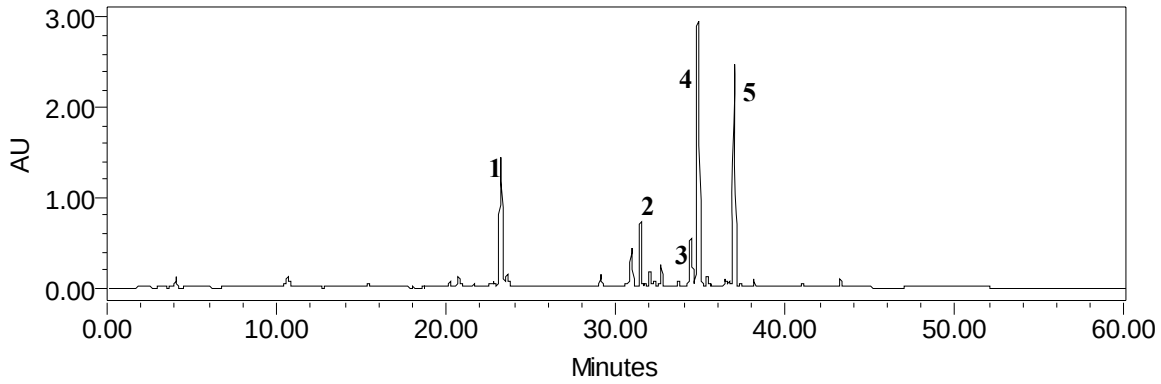
Distribution: This species is found in the in the Northern Cape and in the Free Sate on rocky slopes (Codd, 1985).

Leaves and flowers of *S. namaensis*.



Distribution of S. *namaensis.*

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, 7-*O*-methylepirosmanol, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. namaensis.*

A summary of the HPLC/UV data of the methanol:chloroform extract of *S. namaensis*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.21	330	Rosmarinic acid	15.60
2	31.46	206, 289		9.99
3	34.29	288, 326		4.66
4	34.84	206, 284, 332	Carnosol	30.97
5	36.98	206, 285	Carnosic acid	20.25

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the total phenolic content are given in table which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	36.36	24.39	> 100.0	25.38	16.57	10.63	21.91	190.5

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

Reference

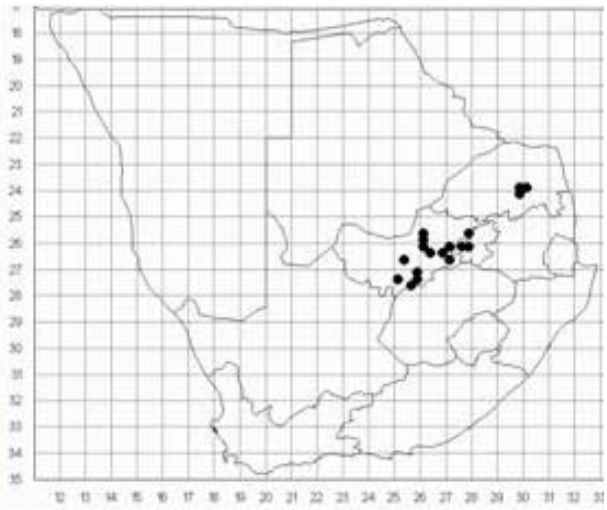
Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

C12. *Salvia radula* Benth.

Botanical description: Perennial herb with one or more erect stems from a woody rootstock and can reach up to 1 m in height or more. Leaves are often crowded and larger near the base of the plant. The calyx is gland-dotted and the corolla is white or pale mauve to blue. This plant is closely related to *S. disermas* but can usually be distinguished by its denser lanate indumentum on lower stems and the undersides of the leaves (Codd, 1985).

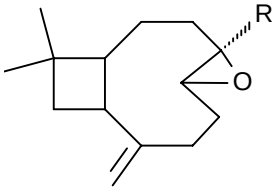
Distribution: This species is found in Gauteng and North-West Province aided by road building and overgrazing of veld (Codd, 1985).

Glandular stem and flowers of *S. radula*.

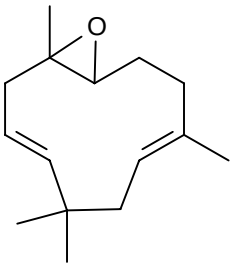


Distribution of *S. radula*

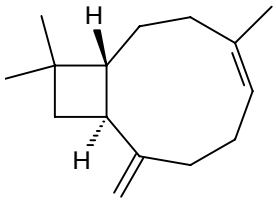
Essential oil composition: The essential oil yield of this plant collected in North-West was 0.004% (w/w, wet biomass) and the major components include β-caryophyllene oxide (22.6%), humulene epoxide (12.6%) followed by isocaryophyllene (8.6%) and spathulenol (7.7%).



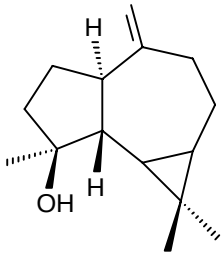
β-Caryophyllene oxide



Humulene epoxide

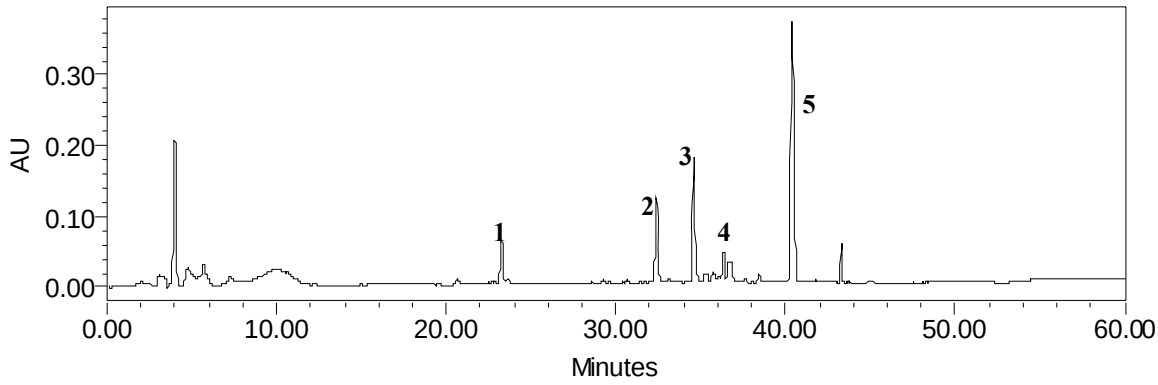


Isocaryophyllene



Spathulenol

HPLC analysis of the solvent extract: Rosmarinic acid, salvigenin and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. radula*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. radula* is displayed below.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.26	238, 329	Rosmarinic acid	3.36
2	32.40	269, 338	Flavone	5.65
3	34.56	235, 277, 329	Salvigenin	8.52
4	36.36	272, 338		4.84
5	40.38	271, 373, 392		20.67

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{+}$	$DPPH^{\cdot}$	MTT	FCM
SE	27.55	9.69	32.10	78.78	13.50	69.26	> 100.0	20.12	55.73
EO	nd	nd	nd	> 100.0	3.91	> 100.0	> 100.0	22.90	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) against bacteria are depicted in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	> 16.00	4.00	1.00	0.06	0.10

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

C13. *Salvia repens* Burch. ex Benth.

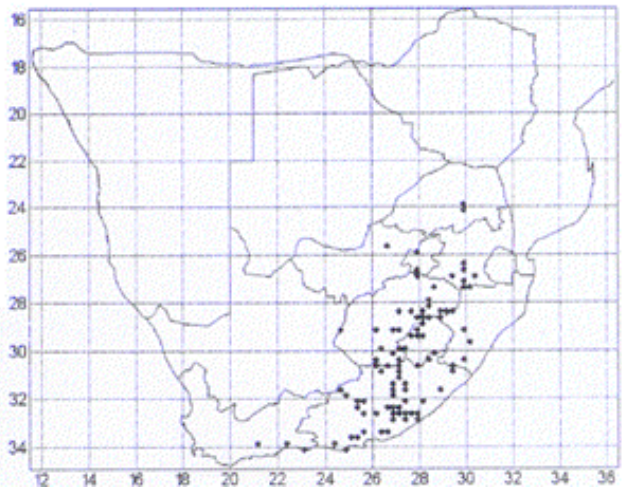
Synonyms: *S. rudis* Benth., *S. incisa* Benth., *S. natalensis* Benth., *S. cooperi* Skan, *S. subssillis* Benth.

Common name: 'Kruipsalie'.

Botanical description: An easy and fast-growing herb, *Salvia repens* has rhizomes, which spread to form small clumps of soft; hairy stems about 400 mm tall. Simple or branched, the stems are square (typical of *Salvia*) and the leaves are formed opposite each other along the stem. The small, two-lipped flowers vary in colour from pure white to pale blue, mauve and purple (Codd, 1985). The main flowering season is during the summer from about October to March.

Distribution: *Salvia repens* is widespread in eastern part of South Africa. It is most common in the grasslands of the highveld and in open woodland (Codd, 1985).

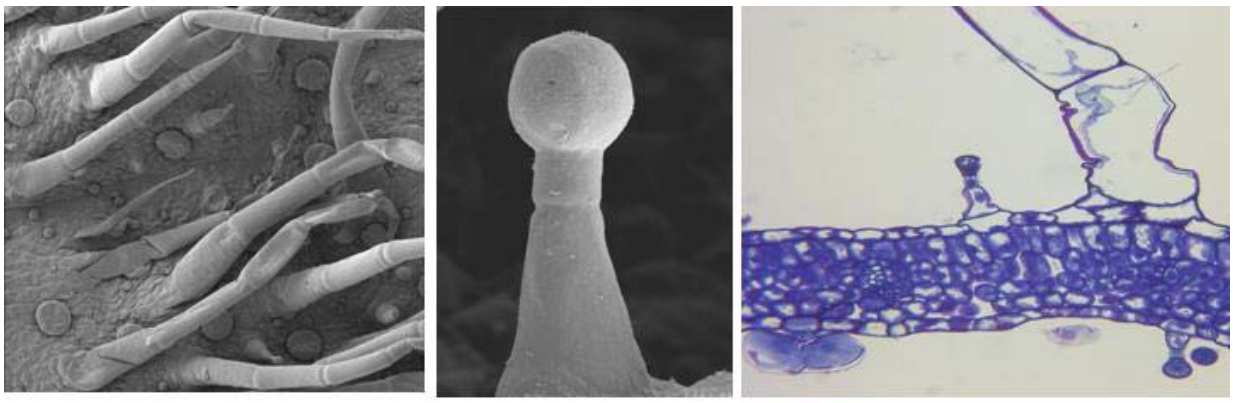
Large leaves and flowers of *S. repens*.



Distribution of *S. repens*.

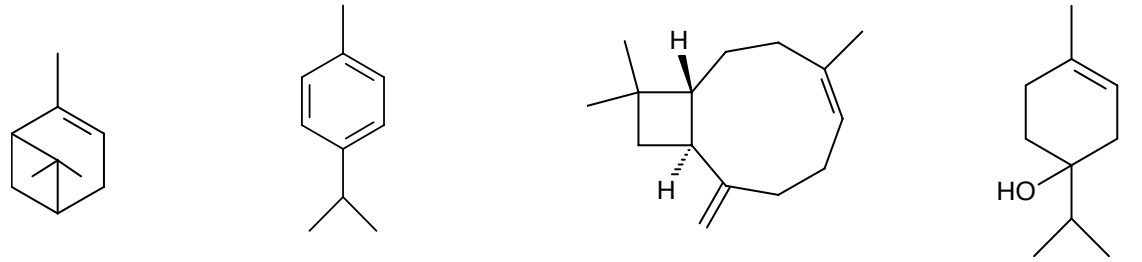
Traditional uses: The leaves have been added to the bath for treating sores, and a decoction of the root has been used for both humans and cattle for treating stomachache and diarrhoea. Smoke from burning the plant can be used as a fumigant (Watt and Breyer-Brandwijk, 1962).

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



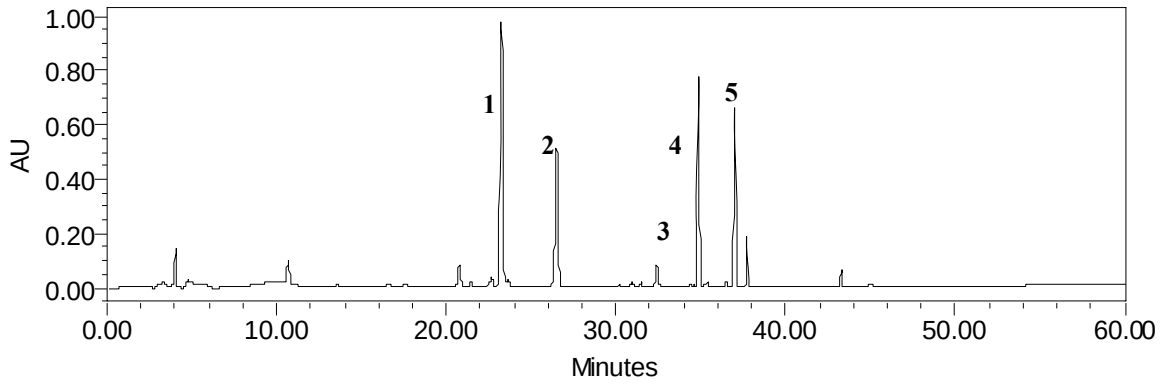
Scanning electron microscopy and light microscopy micrographs of *S. repens*.

Essential oil composition: The essential oil yield of this plant collected from Lady Grey was 0.13% (w/w, wet biomass) and the oil has a predominance of 1,8-cineole (12.8%), *p*-cymene (9.5%) followed by isocaryphyllene (8.6%) and terpinen-4-ol (7.1 %)



1,8-Cineole *p*-Cymene Isocaryphyllene Terpinen-4-ol

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. repens*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. repens*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.24	329	Rosmarinic acid	23.66
2	26.50	328		13.29
3	32.40	240, 285, 331	Flavone	3.10
4	34.86	206, 284, 321	Carnosol	13.40
5	37.02	285, 372	Carnosic acid	13.01

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in table, which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	23.36	43.62	> 100.0	8.25	18.24	15.47	23.24	178.17
EO	nd	nd	nd	28.04	1.65	> 100.0	> 100.0	6.66	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) against bacteria are displayed in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	2.00	3.00	0.03	0.25	0.50

References

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

Watt, J.M., Breyer-Brandwijk, B.N. 1962. *Medicinal and Poisonous Plants of Southern and Eastern Africa*. 2nd edition. E. and S. Livingstone, Edinburg, UK.

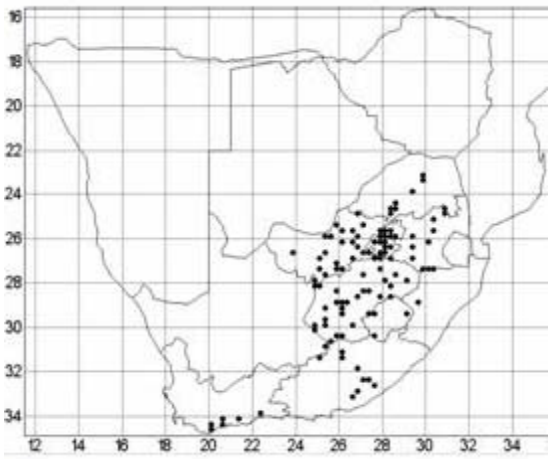
C14. *Salvia runcinata* L.f.

Synonyms: *S. scabra* Benth., *S. monticola* Benth.

Botanical description: Perennial erect herb up to 0.7 m tall with one to several stems from a taproot. Leaves, shortly petiolate or the upper ones sessile, blade runcinate-pinnatipartite to lyrate. The inflorescence has several verticils. The calyx is hispid-scabrid, gland-dotted and the corolla is white or pale blue to mauve or purplish (Codd, 1985).

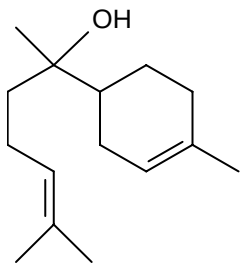
Distribution: Distributed to the Free State and Eastern Cape as far south as Bredasdorp district, rare in the Eastern Cape, KwaZulu-Natal and Lesotho, but also found in Gauteng and North-West Province (Codd, 1985).

Runcinate-pinnatipartite leaves and pale-blue flowers of *S. runcinata*.

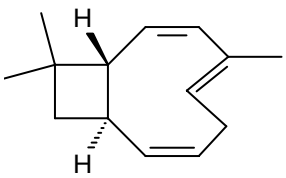


Distribution of *S. runcinata*.

Essential oil composition: The essential oil yield of this plant collected at the Klerkskraal Dam was 0.20% (w/w, wet biomass) and this oil was largely dominated by α-bisabolol (65.5%) and β-caryophyllene (10.5%).



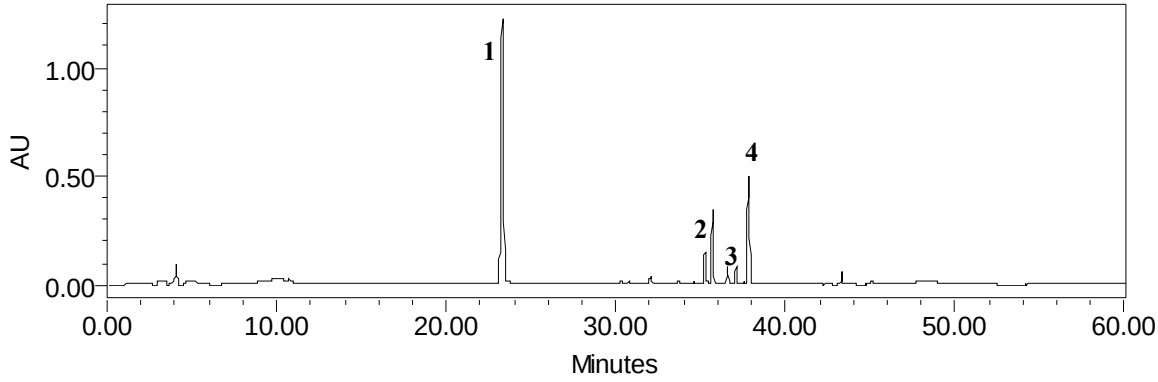
α-Bisabolol



β-Caryophyllene

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, salvigenin, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is

displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. runcinata*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. runcinata*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	23.29	329	Rosmarinic acid	38.40
2	35.25	237, 288, 373		3.55
3	37.10	236, 283, 328	Carnosic acid	3.36
4	37.83	244, 282		14.34

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in the table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	43.42	55.37	> 100.0	16.61	19.42	19.34	22.00	149.34
EO	nd	nd	nd	22.81	1.20	> 100.0	> 100.0	1.79	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) and essential oil (EO) against bacteria are depicted in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	2.00	4.00	0.03	0.25	0.50
EO	> 32.00	> 32.00	2.00	> 32.00	nd

nd: not determined

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

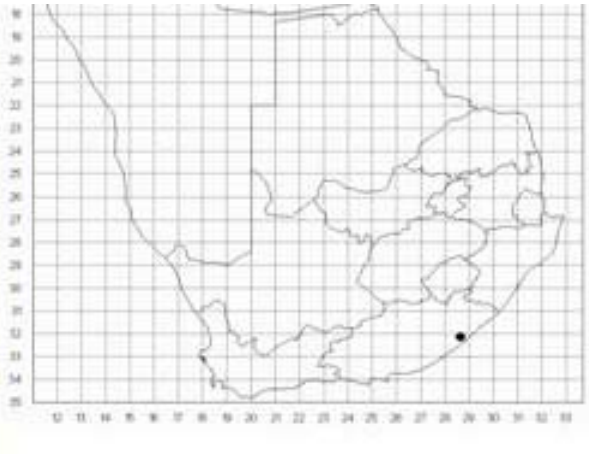
C15. *Salvia schlechteri* Briq.

Synonym: *S. monticola* Benth.

Botanical description: Herbaceous or perennial herb up to 0.3 m tall usually branched from a woody base. Leaves are sessile or subsessile, pinnatisect or pinnatifid and relatively large. The inflorescence is composed of several to many verticils. Calyx is gland-dotted and the corolla, wide-throated is pale blue and white (Codd, 1985). The flowering time starts in January and ends in March.

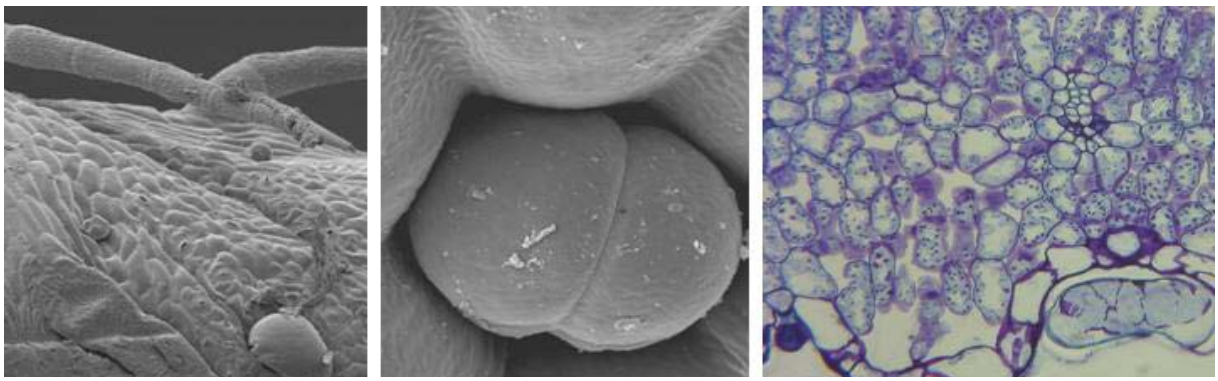
Distribution: This species is generally found in disturbed area and well drained soil, and narrow endemic to Eastern Cape (Codd, 1985).

Pinnatifid or pinnatisect leaves and flowers of *S. schlechteri*.



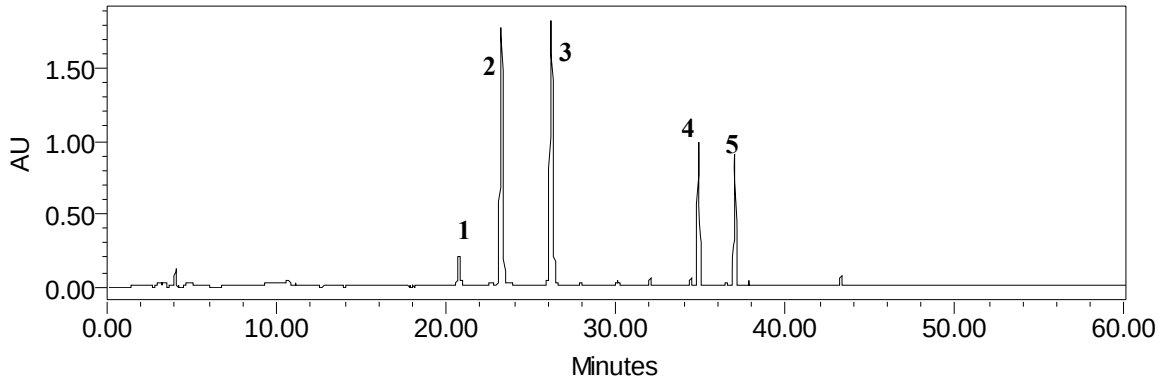
Distribution of *S. schlechteri*.

Leaf trichome types: Both non-glandular and glandular trichomes are present on the leaf. The glandular trichomes are either peltate or capitate.



Scanning electron microscopy and light microscopy micrographs of *S. schlechteri.*

HPLC analysis of the solvent extract: Caffeic acid, rosmarinic acid, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. schlechteri*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. schlechteri*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	20.75	269, 341	Flavone	3.75
2	23.24	330	Rosmarinic acid	26.58
3	26.19	329		31.09
4	34.87	206, 284, 321	Carnosol	10.88
5	37.02	206, 285	Carnosic acid	10.38

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the total phenolic content are given in the table which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{+}$	$DPPH^{\cdot}$	MTT	FCM
SE	54.40	18.37	57.00	> 100.0	17.51	17.51	1.61	28.16	209.18

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) against bacteria are depicted in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	3.00	0.75	0.25	0.50

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

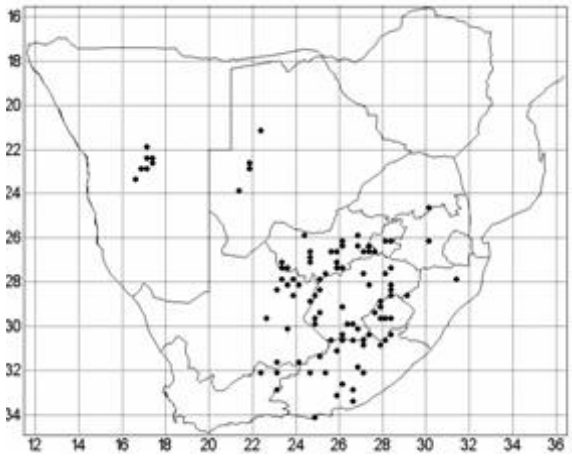
C16. *Salvia stenophylla* Burch. ex Benth.

Synonyms: *S. xerobia* Briq., *S. chlorophylla* Briq, *S. pallida* Dinter

Botanical description: It is a perennial erect herb that can reach 0.6 m tall and usually much branched from a woody taproot. The leaves are shortly petiolated, linear oblong to oblong-lanceolate. The calyx is hispidulous, gland dotted and the corolla is pale blue or mauve (Codd, 1985).

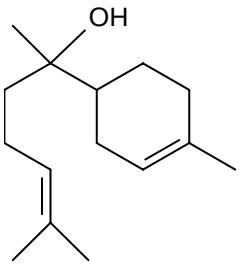
Distribution: The plant is common in Gauteng, Free State and rarely found in KwaZulu-Natal (Codd, 1985).

Pinnatifid or pinnatisect leaves of *S. stenophylla*.

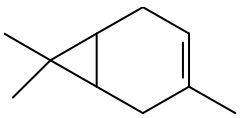


Distribution of *S. stenophylla.*

Essential oil composition: The essential oil yield of this plant collected at the East of Clarens was 0.25% (w/w, wet biomass) and the major components of this oil were α-bisabolol (26.1%) and δ-3-carene (22.6%).

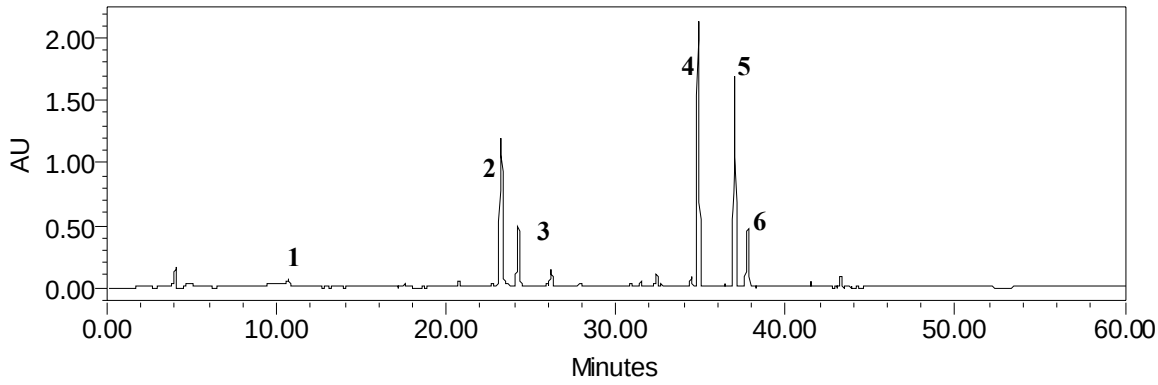


α-Bisabolol



δ-3-Carene

HPLC analysis of the solvent extract: Rosmarinic acid, carnosol, carnosic acid, ursolic acid and betulafolientriol oxide were identified. The HPLC chromatogram is displayed in the figure below, while the retention time, the absorption maxima and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. stenophylla*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. stenophylla*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	10.65	282, 316, 344		5.31
2	23.22	330	Rosmarinic acid	22.17
3	24.23	287, 254, 310		4.10
4	34.86	206, 284, 323	Carnosol	23.60
5	37.00	203, 285	Carnosic acid	25.17
6	37.77	282		6.33

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the essential oil (EO), as well as the total phenolic content are given in the table below.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	43.86	23.74	17.41	> 100.0	6.50	20.84	14.92	12.12	161.28
EO	nd	nd	nd	49.17	4.13	> 100.0	> 100.0	1.98	-

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method; nd: not determined. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract and the essential oil against bacteria are given in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	4.00	2.00	0.03	0.06	0.50
EO	> 32.00	8.00	1.50	> 32.00	nd

nd: not determined

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

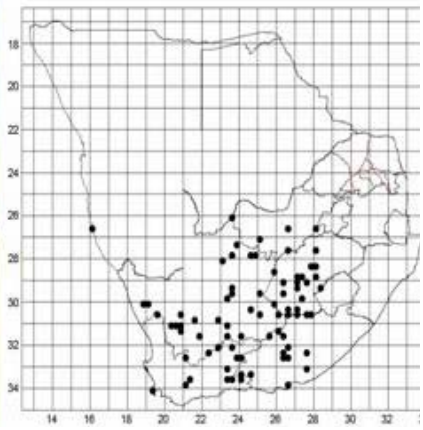
C17. *S. verbenaca* L.,

Synonyms: *Horminum verbenaca* (L.) Mill., *S. cladestina* L., *S. controversa* Ten., *S. cleistogama* De Bary and Paul

Botanical description: It is a perennial short-lived plant with stems arising from woody taproot. The leaves form a dense basal rosette, shortly petiolate to subsessile. The inflorescence is usually branched with many verticils. The calyx is pilose and gland-dotted. The corolla is light blue to purple. Its nearest affinity is with *S. disermas* and *S. radula*.

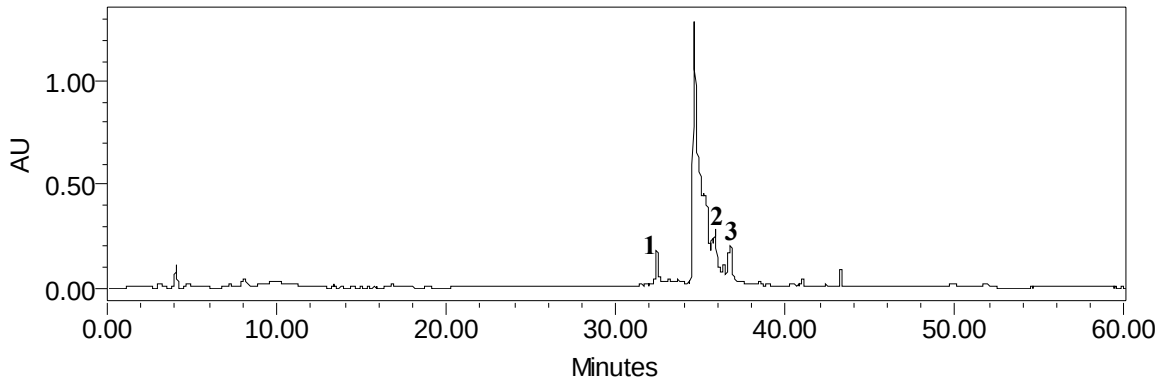
Distribution: This plant is distributed in the central and Western Cape Province and it also occurs in the Free State (Codd, 1985).

Rosette leaves, inflorescence and glandular stem of *S. verbenaca.*



Distribution of *S. verbenaca.*

HPLC analysis of the solvent extract: Salvigenin, ursolic acid and betulafolientriol oxide were identified. *S. verbenaca* was the only species devoid of rosmarinic acid. The HPLC chromatogram is displayed in the figure below, while the retention time, the UV spectra and the percentage area of the major peaks are depicted in the table below.



HPLC chromatogram of the solvent extract of *S. verbenaca*.

A summary of the HPLC-UV data of the methanol:chloroform extract of *S. verbenaca*.

Peak number	Retention time (min)	λ_{max} (nm)	Tentative identification	Area (%)
1	32.41	220, 285, 332		7.46
2	36.77	272, 333		8.17
3	49.82	272, 327		3.09

Biological activities and total phenolic content: The IC_{50} values (in µg/ml) of the *in vitro* biological activities of the solvent extract (SE) and the total phenolic content are given in the table which follows.

	AC			AI	AM	AO		TO	TP
	SF-268	MCF-7	HT-29	5-LOX	^{3}[H]-hyp	$ABTS^{\cdot+}$	$DPPH^{\cdot}$	MTT	FCM
SE	> 100.0	31.50	50.04	> 100.0	23.97	37.47	45.83	20.85	73.29

AC: anticancer activity; AI: anti-inflammatory activity; AM: antimalarial activity; AO: anti-oxidant activity; TO: toxicity profile: TP: total phenolic content; 5-LOX: 5-lipoxygenase assay; [^{3}H]-hyp; hypoxanthine isotope method; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay; FCM: Folin-Ciocalteu method. NB: the anticancer activity was determined using the SRB assay, while the anti-oxidant activity was investigated using the ABTS and the DPPH methods.

The MIC values (mg/ml) of the solvent extract (SE) against bacteria are displayed in the table below.

	Pathogens				
	E. coli	*K. pneumoniae*	*B. cereus*	*S. aureus*	*M. tuberculosis*
SE	8.00	2.00	2.00	3.00	0.10

Reference

Codd, L.E.W. 1985. *Lamiaceae*: *Flora of Southern Africa*. **28,** Botanical Research Institute, Pretoria.

Appendix D: ^{1}H and ^{13}C spectra of compounds isolated from *Salvia radula*

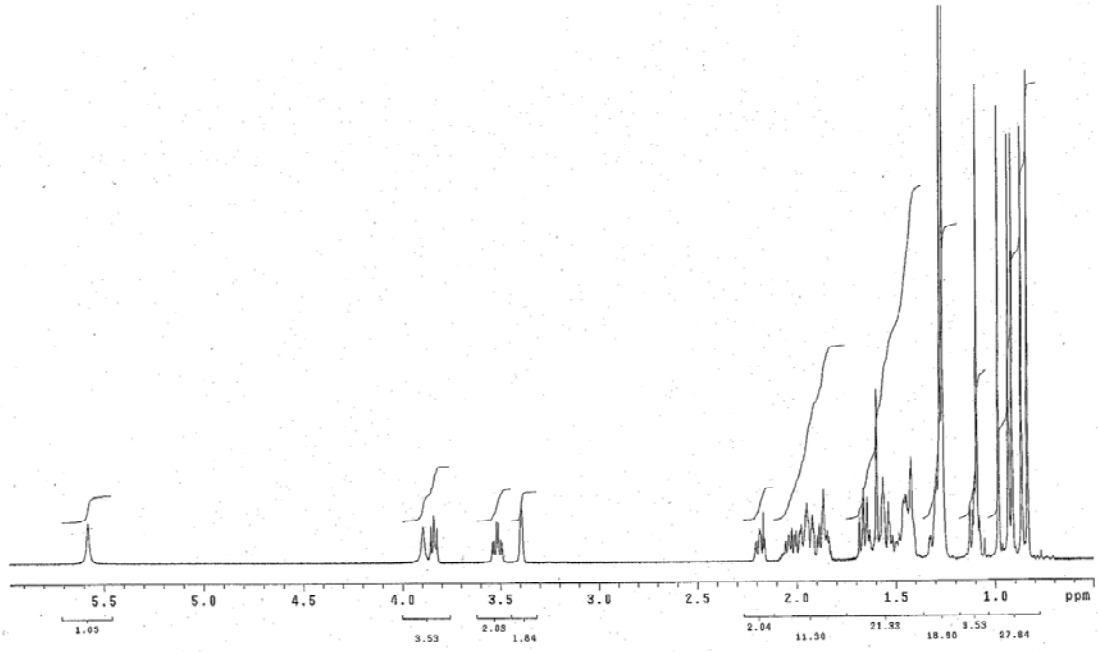


Figure D1 ^{1}H spectrum of betulafolientriol oxide.

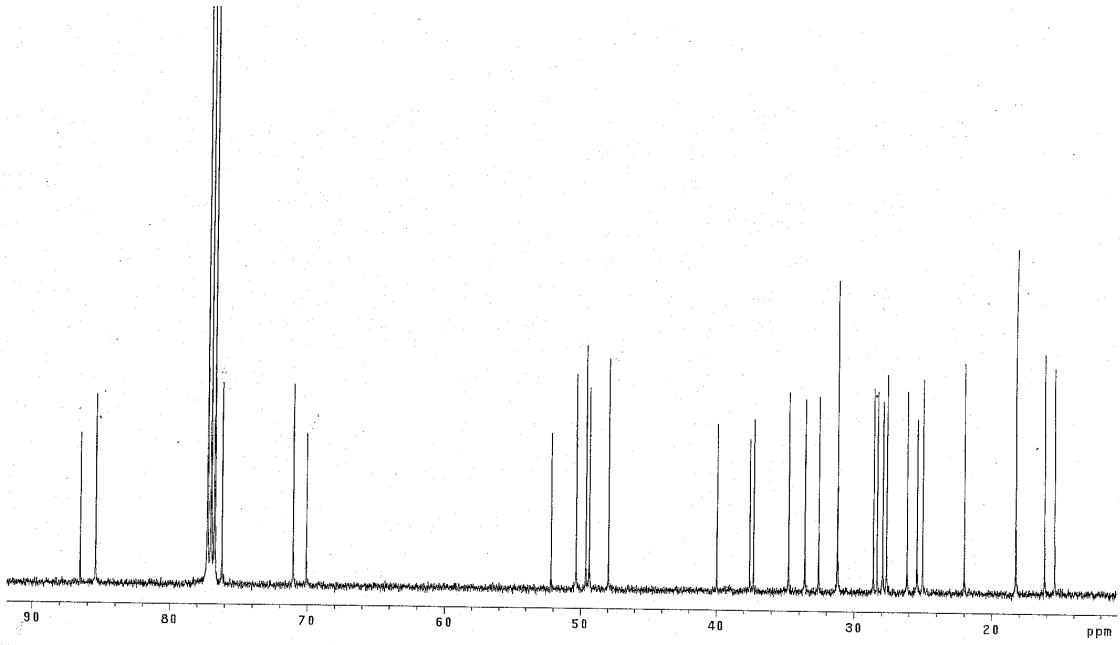


Figure D2 ^{13}C spectrum of betulafolientriol oxide.

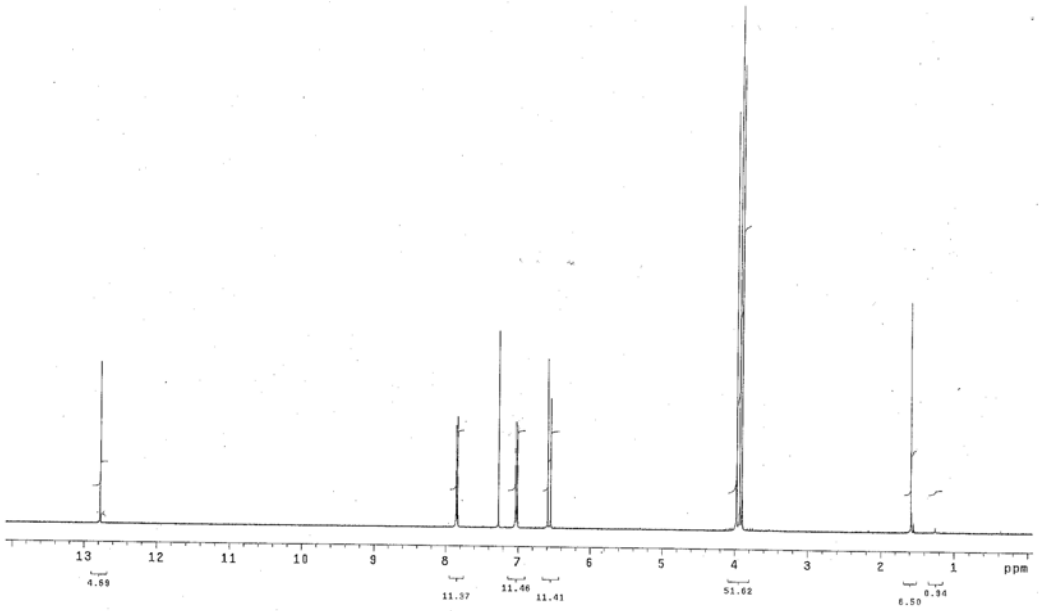


Figure D3 ^{1}H spectrum of salvigenin.

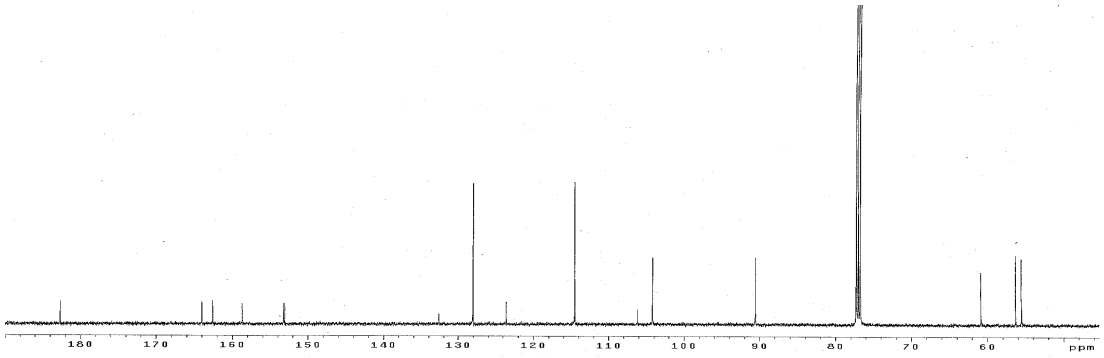


Figure D4 ^{13}C spectrum of salvigenin.

Appendix E: ^{1}H and ^{13}C spectra of compounds isolated from *Salvia chamelaeagnea*

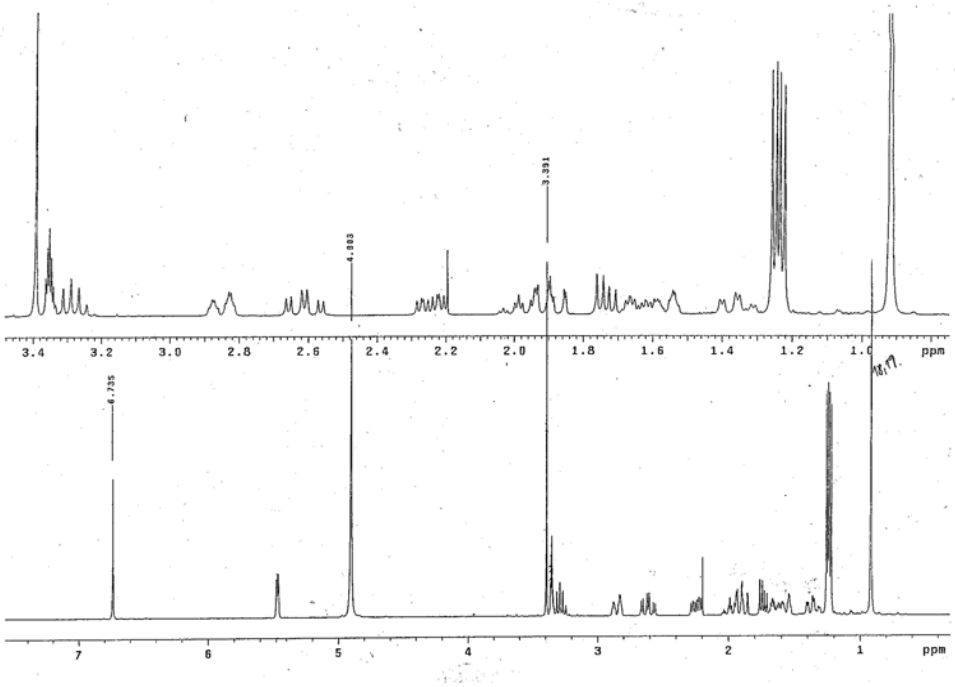


Figure E1 ^{1}H spectrum of carnosol.

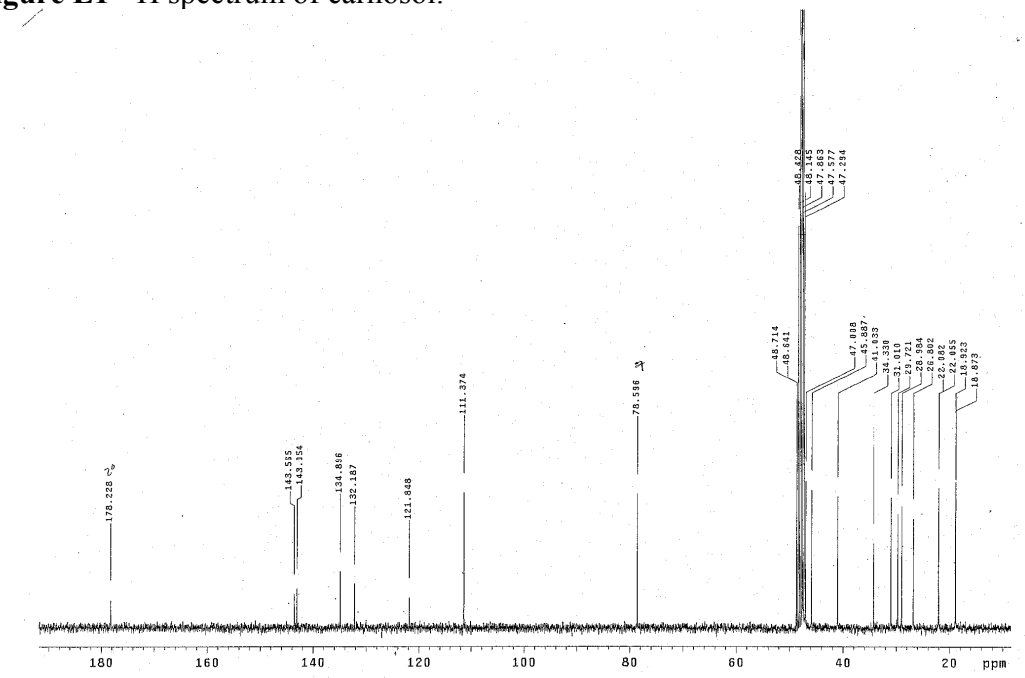


Figure E2 ^{13}C spectrum of carnosol.

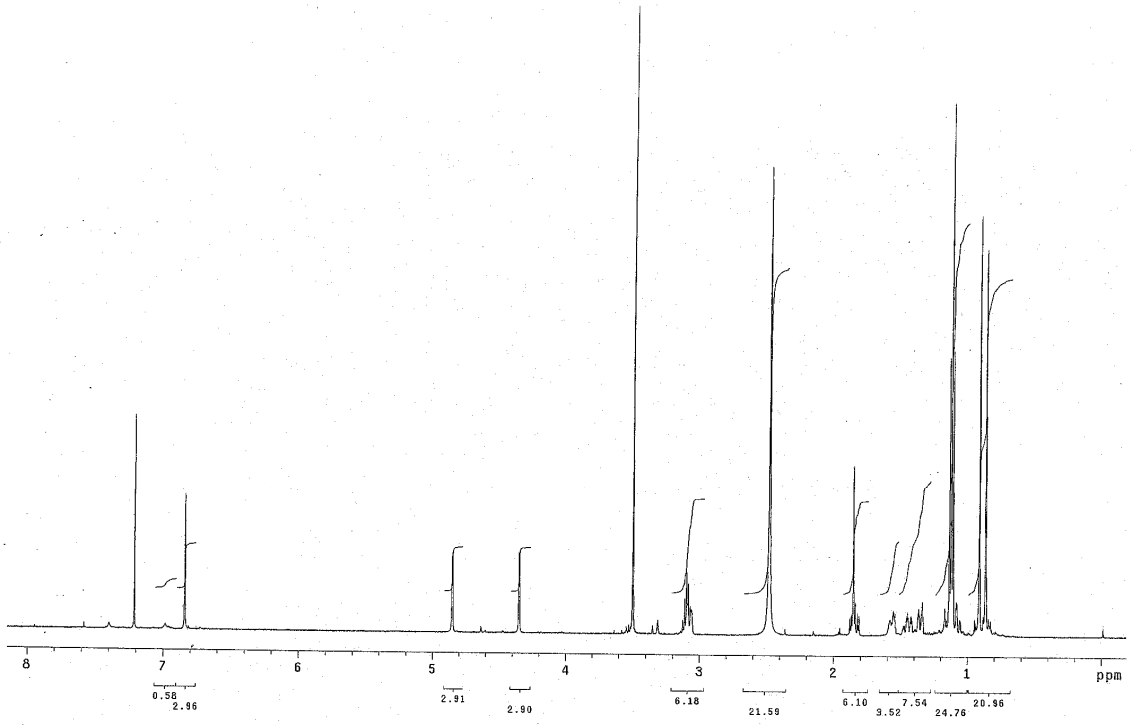


Figure E3 ^{1}H spectrum of 7-*O*-methylepirosmanol.

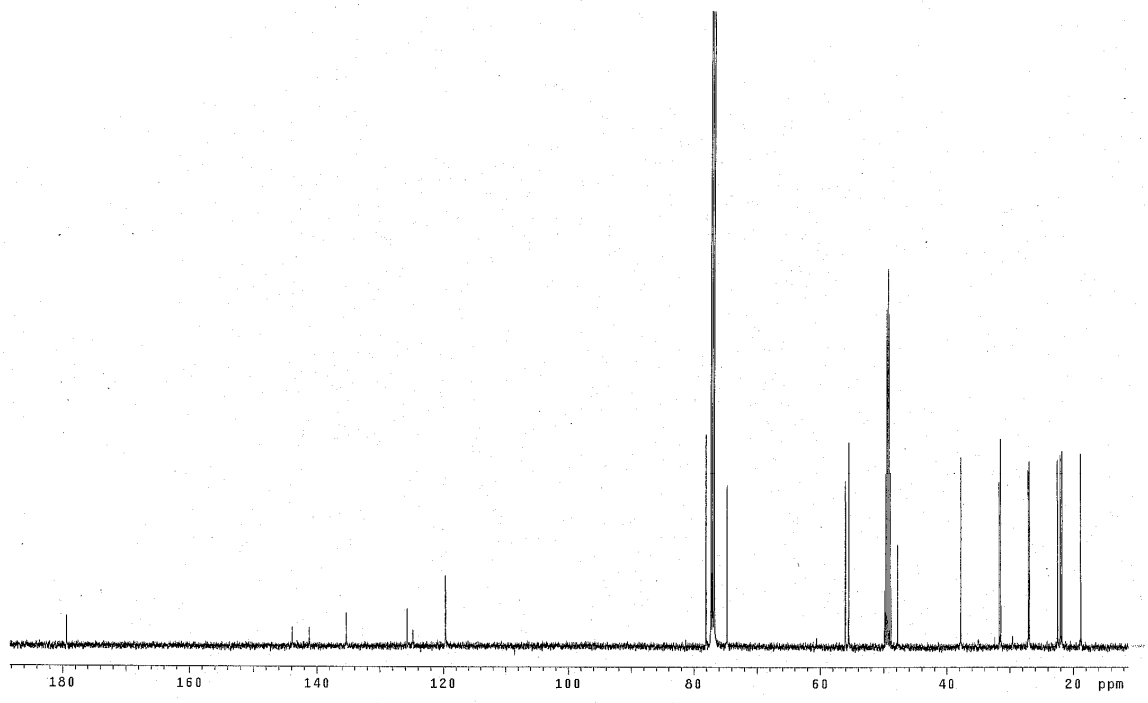


Figure E4 ^{13}C spectrum of 7-*O*-methylepirosmanol.

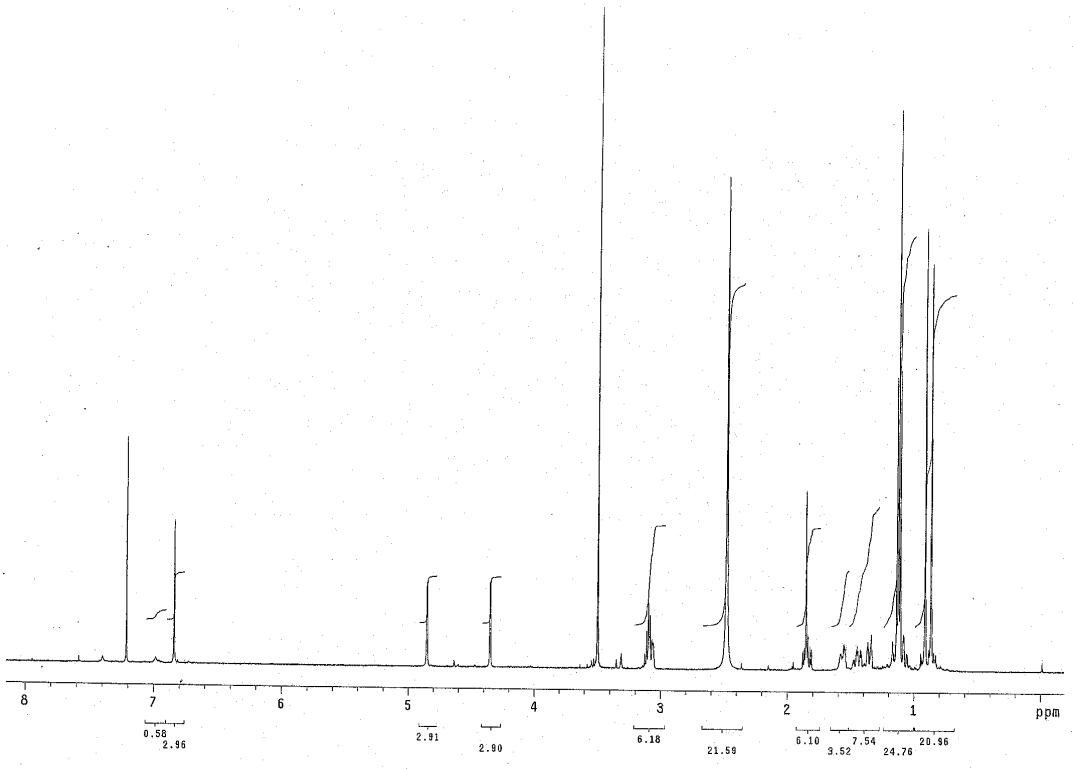


Figure E5 ^{1}H spectrum of ursolic/oleanolic acid.

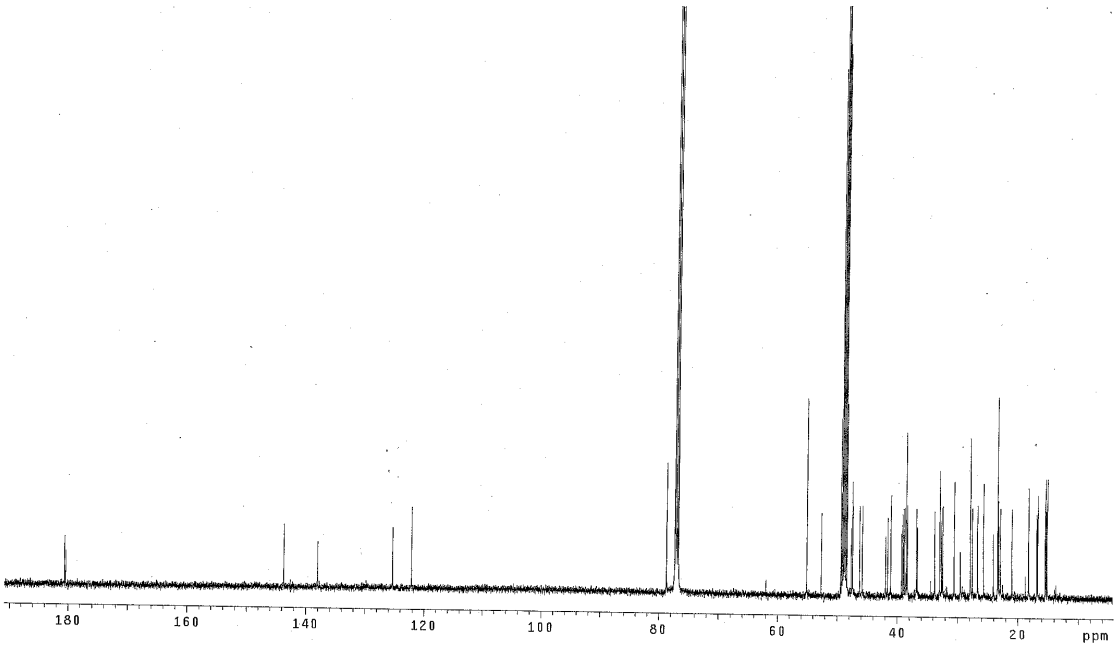


Figure E6 ^{13}C spectrum of ursolic/oleanolic acid.

Appendix F: Seasonal variation in essential oil composition of *Salvia africana-caerulea*, *S. africana-lutea* and *S. lanceolata*

Table F-1 Percentage of the components identified in the essential oil of *Salvia africana-caerulea* over a one year period.

Components	RI	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
α-Thujene	924	tr	0.1	1.6	0.1	0.1	tr	1.2	0.3	0.1	0.4	0.1	0.1
Benzaldehyde	927	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
α-Pinene	**930**	**1.7**	**4.2**	**tr**	**3.4**	**4.0**	**2.6**	**tr**	**3.9**	**3.7**	**8.2**	**4.1**	**4.7**
Camphene	938	0.7	0.1	0.1	0.1	0.1	tr	0.1	0.1	0.1	0.1	0.1	0.1
Sabinene	958	0.2	0.2	tr	0.3	0.4	0.2	tr	0.2	0.3	0.1	tr	0.4
1-Octen-3-ol	961	0.2	0.2	tr	0.3	0.4	0.2	0.2	0.2	tr	0.1	tr	0.4
β-Pinene	963	tr	0.3	tr	tr	tr	tr	0.2	0.3	1.0	2.9	1.3	tr
Myrcene	**975**	**0.6**	**0.8**	**0.6**	**0.7**	**0.6**	**0.6**	**tr**	**0.8**	**0.6**	**10.7**	**0.2**	**0.8**
α-Phellandrene	995	0.6	0.5	0.4	0.5	0.5	0.3	0.3	0.3	0.2	0.8	-	0.6
p-Cymene	1003	tr	0.1	0.1	0.1	tr	0.2	0.1	0.6	0.2	0.2	1.5	0.2
o–Cymene		0.2	0.4	0.7	0.4	tr	0.2	0.1	0.3	0.2	0.2	-	0.6
1,8-Cineole	1005	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.5	tr
β-Phellandrene	**1005**	**0.4**	**0.8**	**tr**	**0.4**	**1.0**	**0.4**	**tr**	**0.1**	**1.2**	**7.8**	**-**	**0.8**
Limonene	**1009**	**24.9**	**31.4**	**23.4**	**27.3**	**28.5**	**21.4**	**10.2**	**29.2**	**31.5**	**5.8**	**1.5**	**32.6**
cis-β-Ocimene	1017	0.1	0.1	0.1	0.2	tr	0.1	tr	0.3	0.2	3.1	tr	0.2
trans-β-Ocimene	1027	0.3	tr	tr	0.1	tr	tr	tr	0.1	0.2	1.0	tr	tr
γ-Terpinene	1035	tr	0.1	tr	0.1	0.1	0.1	tr	0.1	0.1	0.2	-	0.1
trans-Sabinene hydrate	1037	tr	0.1	tr	0.1	0.1	0.1	tr	0.1	0.2	0.2	tr	0.1
cis-Sabinene hydrate	1066	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.1	tr
Linalol	1074	1.5	0.4	1.6	0.6	0.4	0.3	0.4	0.4	0.4	0.3	0.3	0.4
trans-p-2-Menthen-1-ol	1074	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.6	tr
Camphor	1095	0.4	0.2	0.1	0.3	0.2	0.3	0.5	tr	tr	0.3	-	0.2
δ-Terpineol	1134	-	-	-	-	-	-	-	-	-	-	tr	-

Components	RI	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
Terpinen-4-ol	**1148**	**8.3**	**1.4**	**0.3**	**0.4**	**3.2**	**2.2**	**7.3**	**1.7**	**1.1**	**1.1**	**0.5**	**1.1**
α-Terpineol	1159	1.2	0.4	1.5	1.1	0.4	0.2	1.4	0.2	0.1	0.5	0.7	0.4
trans-Carveol	1189	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
Carvone	1206	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
cis-Jasmone	1372	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
α–Gurjunene	1400	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr
β-Caryophyllene	1414	0.7	0.4	0.5	0.4	0.5	0.2	0.7	0.3	0.4	0.3	1.9	0.4
β-Gurjunene*	1426	6.1	1.4	1.3	2.3	3.8	3.9	6.7	2.0	1.3	4.3	0.7	1.0
Aromadendrene	1428	tr	0.5	0.7	tr	tr	tr	2.2	tr	tr	tr	0.9	0.6
Eudesmadiene*	1435	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.8	tr
α-Humulene	1447	tr	0.3	0.6	1.4	tr	0.9	tr	1.3	0.5	1.8	1.2	0.4
allo-Aromadendrene	1456	1.5	0.9	0.7	0.1	2.5	0.7	1.3	0.2	1.4	0.5	tr	0.9
Viridiflorene	1487	0.1	1.1	2.2	1.3	1.0	1.8	2.3	1.0	1.5	1.1	0.2	1.2
α–Muurolene	1494	1.0	0.4	1.4	0.6	2.2	2.8	1.1	0.4	0.5	1.1	-	0.4
γ–Cadinene	1500	0.6	1.0	0.9	1.2	0.9	0.9	tr	2.4	1.3	0.3	1.5	2.0
***trans*-Calamenene**	**1505**	**0.2**	**0.8**	**2.8**	**3.4**	**4.4**	**8.9**	**tr**	**0.7**	**0.3**	**3.4**	**0.3**	**1.0**
trans-Nerolidol	1549	1.2	1.9	2.2	2.3	2.5	2.4	2.0	2.4	2.3	0.7	1.4	1.9
β-Caryophyllene alcohol	1550	1.8	2.3	2.9	3.0	2.6	3.1	1.6	4.0	3.6	1.4	1.4	2.2
Spathulenol	**1551**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**29.1**	**-**
β-Caryophyllene oxide	**1561**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**14.6**	**-**
Globulol	1566	-	-	-	-	-	-	-	-	-	-	tr	-
Viridiflorol	**1569**	**11.1**	**21.0**	**24.4**	**22.8**	**18.9**	**20.1**	**14.2**	**22.8**	**21.2**	**1.6**	**6.5**	**20.6**
Ledol	**1580**	**6.4**	**2.6**	**3.7**	**3.7**	**3.7**	**5.6**	**7.5**	**3.4**	**3.1**	**1.9**	**tr**	**2.2**
epi-Cubenol	1600	1.3	0.8	1.3	1.3	1.4	2.0	1.1	1.2	1.2	0.8	tr	0.8
T- Cadinol	1616	0.7	3.4	2.5	3.8	3.4	3.4	2.5	3.9	3.5	1.3	3.0	3.3
β-Eudesmol	1620	0.4	0.8	3.2	1.0	0.8	0.7	0.7	0.5	0.6	3.8	0.7	0.8
α–Eudesmol	1634	1.4	1.4	3.0	2.3	2.2	2.3	2.7	1.5	1.4	7.7	2.0	1.5
% Identification		**75.8**	**82.8**	**84.8**	**87.4**	**90.8**	**89.1**	**68.6**	**87.2**	**85.4**	**76.0**	**78.0**	**85.0**

RI: Relative retention indices calculated against *n*-alkanes. - : not present, tr: trace (< 0.05 %); *: determined by mass spectra only.

Table F-2 Percentage of the components identified in the essential oil of *Salvia africana-lutea* over a one year period.

Components	RI	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sept	Oct	Nov	Dec
α-Thujene	924	0.5	0.1	0.4	0.4	0.3	0.3	0.5	0.4	0.2	0.2	0.6	0.1
α-Pinene	**930**	**11.0**	**0.5**	**7.1**	**8.6**	**11.6**	**5.0**	**11.9**	**9.8**	**4.6**	**3.2**	**6**	**1.1**
Camphene	938	tr	0.1	tr	tr	0.1	tr	0.1	0.1	0.1	0.1	0	0.1
Sabinene	958	tr	0.3	1.4	1.9	3.9	2.7	0.9	0.1	0.1	0.7	0.9	0.1
1-Octen-3-ol	961	tr	0.3	1.4	1.9	3.8	2.7	0.9	0.1	0.1	0.7	0.9	0.1
β-Pinene	963	3.4	0.2	tr	tr	tr	1.8	2.9	3.8	1.9	tr	0.2	0.9
Myrcene	**975**	**10.7**	**3.7**	**5.0**	**2.6**	**4.3**	**5.9**	**5.3**	**8.4**	**2.7**	**5.4**	**11.5**	**2.2**
α-Phellandrene	995	0.6	0.2	0.8	0.4	0.9	1.1	1.0	0.8	0.8	0.7	-	0.6
δ–3–Carene	**1000**	**1.9**	**3.9**	**1.1**	**7.8**	**1.5**	**8.3**	**8.0**	**3.5**	**4.4**	**0.1**	**-**	**6.8**
***o*–Cymene**	**1000**	**10.3**	**0.4**	**8.1**	**3.0**	**7.1**	**0.6**	**3.4**	**0.7**	**0.5**	**0.3**	**-**	**1.1**
***p*-Cymene**	**1003**	**2.6**	**0.2**	**1.3**	**0.7**	**0.4**	**0.3**	**0.4**	**0.2**	**0.2**	**0.1**	**7.6**	**0.3**
1,8-Cineole	**1005**	**5.3**	**0.5**	**3.2**	**2.6**	**2.4**	**4.0**	**4.9**	**3.5**	**2.4**	**2.5**	**1.9**	**3.3**
β-Phellandrene	**1005**	**5.3**	**0.5**	**3.2**	**2.6**	**2.4**	**4.0**	**4.9**	**3.5**	**2.4**	**2.5**	**-**	**3.3**
Limonene	**1009**	**tr**	**3.4**	**tr**	**2.8**	**tr**	**6.0**	**3.7**	**5.4**	**3.7**	**3.3**	**1.6**	**5.3**
***cis*-β-Ocimene**	**1017**	**1.4**	**3.0**	**1.3**	**tr**	**0.5**	**0.5**	**0.5**	**1.7**	**0.2**	**2.1**	**5.4**	**0.2**
trans-β-Ocimene	1027	0.5	1.1	0.6	tr	0.1	0.2	0.1	0.6	0.2	0.7	1.8	0.1
γ-Terpinene	1035	tr	0.4	0.1	tr	0.1	0.2	0.2	0.1	0.2	0.1	-	0.1
trans-Sabinene hydrate	1037	tr	0.4	0.1	tr	0.1	0.2	0.2	0.1	0.2	0.1	0	4.7
Linalol	1074	0.6	0.2	0.3	0.2	tr	tr	tr	0.4	0.7	0.4	0.8	0.8
Camphor	1095	0.2	0.4	0.5	0.4	tr	tr	tr	0.3	0.2	0.4	0.2	0.3
Terpinen-4-ol	1148	1.3	0.1	0.5	0.7	tr	tr	tr	0.2	0.2	tr	0.6	1.6
α-Terpineol	1159	tr	0.3	0.1	tr	tr	tr	tr	tr	0.2	tr	0.8	0.3
1-Decanol	1259	0.8	tr	0.4	0.4	0.4	0.6	0.6	0.8	0.5	0.5	-	1.4
Bornyl acetate	1265	0.5	tr	tr	0.6	tr	tr	tr	tr	0.1	tr	-	0.2
α–Gurjunene	**1400**	**0.5**	**0.6**	**4.7**	**5.0**	**4.6**	**4.3**	**2.5**	**0.7**	**0.8**	**0.4**	**1.1**	**0.9**
β-Caryophyllene	1414	2.7	2.0	2.4	1.5	1.1	0.8	0.9	2.1	4.3	2.6	3.4	3.4
β-Gurjunene*	1426	1.0	3.3	tr	tr	0.2	tr	0.2	2.8	1.5	1.0	0.3	1.2
Aromadendrene	1428	1.0	1.1	1.2	0.7	1.4	0.6	0.8	1.4	1.4	0.9	2.7	0.6
α-Humulene	1447	1.1	1.1	2.4	2.4	1.3	1.8	1.8	1.1	1.9	1.5	1.9	1.7
trans-β-Farnesene	1455	tr	0.4	tr	tr	0.7	tr	0.4	0.9	0.2	0.4	tr	0.1

Components	**RI**	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sept**	**Oct**	**Nov**	**Dec**
allo-Aromadendrene	1456	tr	0.4	tr	tr	0.7	tr	0.4	0.9	0.2	0.4	1.3	0.1
Germacrene-D	1474	0.5	0.9	1.4	0.5	0.4	0.3	0.7	0.5	0.6	1.0	tr	0.8
Bicyclogermacrene	1487	2.2	2.3	1.0	0.8	0.4	0.5	0.2	0.1	0.2	0.5	-	0.4
Viridiflorene	1487	-	tr	tr	tr	tr		-	-	-	-	1.3	-
β-Bisabolene	1495	0.5	1.8	0.9	0.5	0.5	0.4	0.5	0.5	0.8	3.4	1.3	0.8
δ–Cadinene	**1505**	**0.4**	**2.3**	**0.6**	**0.5**	**0.5**	**0.4**	**0.4**	**0.5**	**0.6**	**0.4**	**4.7**	**0.5**
trans-Calamenene	1505	tr	tr	tr	tr	tr	tr	tr	tr	tr	tr	0.9	tr
Elemol	1530	1.6	0.9	1.2	1.3	0.6	0.7	0.8	0.7	1.4	1.8	3.5	2.2
trans-Nerolidol	1549	0.2	0.7	0.7	0.3	0.5	0.2	0.5	0.7	0.6	0.6	2.0	0.3
β-Caryophyllene alcohol	1550	0.2	0.7	0.4	0.3	0.5	0.2	0.5	0.7	0.6	0.6	2.0	0.3
Spathulenol	**1551**	**3.3**	**2.8**	**6.3**	**5.6**	**10.9**	**2.3**	**4.3**	**6.8**	**6.7**	**3.8**	**1.9**	**3.1**
β-Caryophyllene oxide	**1561**	**2.3**	**2.7**	**4.1**	**2.8**	**2.4**	**1.9**	**1.9**	**2.8**	**3.5**	**3.9**	**0.8**	**2.6**
Humulene epoxide*	1579	0.5	2.6	0.8	0.9	0.4	0.7	0.7		tr	1.3	3.3	0.7
epi-Cubenol	1600	-	-	-	-	-	-	-	-	-	-	1.8	-
T- Cadinol	1616	1.3	2.2	1.7	2.7	2.3	3.2	1.8	1.5	2.3	2.2	1.9	1.9
γ-Eudesmol		0.9	4.0	2.4	1.5	2.6	2.9	2.8	2.4	2.2	3.3	-	3.6
β-Eudesmol	**1620**	**3.2**	**8.9**	**2.1**	**4.6**	**4.7**	**1.4**	**5.3**	**1.8**	**9.9**	**2.1**	**1.7**	**12.7**
α–Eudesmol	**1634**	**5.0**	**10.6**	**8.1**	**9.5**	**10.4**	**10.9**	**9.3**	**8.5**	**12.6**	**9.1**	**tr**	**12.9**
% Identification		**85.3**	**72.5**	**79.3**	**79.0**	**87.1**	**77.9**	**87.1**	**81.9**	**79.1**	**65.3**	**78.6**	**85.8**

RI: Relative retention indices calculated against *n*-alkanes. - : not present, tr: trace (< 0.05 %); *: determined by mass spectra only.

Table F-3 Percentage of the components identified in the essential oil of *Salvia lanceolata* over a one year period.

Components	RI	Jan	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Tricyclene	921											
α-Thujene	924	0.4	0.5	0.5	0.4	0.1	0.1	0.2	0.4	0.4	-	0.2
Benzaldehyde	927	-	-	-	-	-	-	-	-	-	0.5	-
α-Pinene	930	3.8	tr	tr	0.3	1.2	0.8	1.4	0.5	2.7		0.3
Camphene	938	0.1	tr	tr	tr	tr	1.1	tr	tr	0.1	2.6	0.1
Sabinene	958	1.9	0.3	0.3	0.2	0.3	0.7	0.8	0.1	1.9	tr	0.1
1-Octen-3-ol	961	1.9	0.3	0.3	0.2	0.3	0.7	0.8	0.1	1.9		0.1
β-Pinene	963	1.8	tr	tr	0.6	0.8	0.8	2.2	0.7	1.3	1.4	0.1
Myrcene	**975**	**3.9**	**0.7**	**0.1**	**1.6**	**1.0**	**3.0**	**1.5**	**2.0**	**6.1**	**-**	**0.1**
α-Phellandrene	995	0.1	tr	0.1	tr	0.2	0.3	0.2	tr	0.1	-	0.1
δ–3–Carene	1000	0.1	tr	tr	0.1	0.2	0.1	0.5	0.1	0.1	-	0.4
o–Cymene		0.9	tr	tr	tr	0.5	0.8	0.3	tr	1.0	-	tr
p-Cymene	1003	1.1	1.0	0.4	0.9	0.5	0.2	0.7	1.4	0.5	-	0.2
1,8-Cineole	1005	0.7	1.0	0.5	1.3	1.2	1.0	0.5	1.0	1.0	1.1	0.6
β-Phellandrene	1005	0.7	1.0	0.5	0.7	1.2	1.0	1.4	1.0	1.0	1.8	0.6
Limonene	1009	3.6	tr	tr	tr	tr	1.6	2.1	tr	1.9		tr
cis-β-Ocimene	1017	0.8	0.1	tr	1.1	1.5	1.9	3.1	1.6	2.5	1.8	tr
trans-β-Ocimene	1027	0.4	tr	tr	0.7	1.0	1.3	1.6	1.5	1.9	tr	tr
γ-Terpinene	1035	tr	tr	tr	tr	0.6	tr	0.1	0.1	0.1	tr	tr
trans-Sabinene hydrate	1037	tr	tr	tr	tr	0.4	tr	0.2	0.1	0.1	-	tr
Linalol	1074	0.6	tr	0.3	0.8	0.7	0.3	0.2	tr	1.1	tr	0.2
Camphor	1095	0.4	tr	0.1	0.4	0.7	0.3	0.2	0.3	tr	-	0.4
Terpinen-4-ol	1148	0.2	1.0	0.7	0.5	0.6	0.6	0.8	0.7	0.8	-	0.4
α-Terpineol	1159	tr	0.2	0.5	0.1	0.4	0.5	0.9	0.1	0.2	1.0	0.3
Bornyl acetate	1265	tr	tr	1.1	tr	tr	0.5	tr	tr	tr	-	tr
β-Bourbonene	1373	0.3	1.5	1.1	0.8	2.1	1.2	1.2	1.0	1.3	-	0.8
β–Elemene	1388	0.7	2.0	1.3	2.2	1.8	1.0	1.1	2.6	0.9	-	0.6
α–Gurjunene	1400	0.3	0.4	0.9	0.2	0.3	tr	tr	0.2	0.3	-	0.9
β-Caryophyllene	**1414**	**12.9**	**13.1**	**3.1**	**19.2**	**18.6**	**17.8**	**17.0**	**18.4**	**12.7**	**-**	**0.7**
β-Gurjunene*	**1426**	**1.6**	**1.8**	**1.1**	**1.5**	**1.2**	**2.3**	**1.4**	**1.5**	**0.7**	**5.7**	**1.8**
Aromadendrene	1428	-	2.9	0.5	3.2	0.2			1.0	0.4	0.1	0.6
γ-Elemene*	1430	0.5	2.0	0.9	0.6	2.0	1.0	1.3	1.2	3.4	-	0.6

Components	RI	Jan	Mar	Apr	May	Jun	July	Aug	Sep	Oct	Nov	Dec
Geranyl acetone	**1434**	**0.4**	**0.8**	**1.9**	**0.6**	**2.0**	**tr**	**0.9**	**5.2**	**5.2**	**-**	**-**
α-*trans*-Bergamotene	1434	-	-	-	-	-	-	-	-	-	0.8	-
α-Humulene	**1447**	**5.9**	**8.4**	**2.7**	**1tr**	**9.7**	**8.5**	**8.6**	**8.5**	**5.5**	**tr**	**1.6**
trans-β-Farnesene	1455	-	-	-	-	-	-	-	-	-	4.7	-
allo-Aromadendrene	1456	0.9	0.5	0.5	0.8	0.9	1.0	1.1	0.9	0.6	-	0.6
Germacrene-D	1474	2.2	1.4	0.3	2.7	2.2	1.9	4.3	1.2	3.0	-	1.2
ar-Curcumene	1475	-	-	-	-	-	-	-	-	-	2.4	-
Bicyclogermacrene	**1487**	**4.8**	**1.3**	**1.0**	**3.4**	**6.0**	**5.4**	**7.9**	**2.5**	**4.7**	**-**	**1.8**
β-Bisabolene	1495	0.9	1.3	0.3	0.9	0.8	1.0	0.9	0.9	0.8	0.2	0.6
γ–Cadinene	1500	1.1	0.7	0.6	1.0	0.8	1.0	1.1	0.6	0.8	-	0.3
trans-Nerolidol	1549	0.7	0.9	0.8	0.8	0.7	3.1	0.9	4.6	0.8	-	0.8
Spathulenol	**1551**	**6.5**	**4.3**	**10.6**	**3.1**	**2.6**	**2.8**	**4.0**	**1.9**	**2.4**	**2.2**	**0.7**
β-Caryophyllene oxide	**1561**	**3.4**	**7.1**	**21.2**	**3.3**	**2.2**	**2.0**	**1.8**	**2.4**	**1.4**	**18.3**	**10.3**
Globulol	**1566**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**14.3**	**-**
Viridiflorol	1569	-	-	-	-	-	-	-	-	-	0.6	-
Ledol	**1580**	**-**	**4.1**	**2.9**	**11.7**	**4.5**	**3.6**	**3.7**	**4.1**	**4.3**	**5.9**	**-**
Humulene epoxide*	**1579**	**2.8**	**6.7**	**3.6**	**4.6**	**4.5**	**4.4**	**4.0**	**4.3**	**6.5**	**5.2**	**10.8**
epi-Cubenol	1600	0.8	1.4	0.4	0.9	0.7	0.8	tr	1.0	tr		0.8
T- Cadinol	1616	0.7	0.8	1.3	0.6	0.7	0.9	1.6	0.2	tr	0.6	1.4
δ-Cadinol	1618	-	-	-	-	-	-	-	-	-	1.9	-
β-Eudesmol	**1620**	**0.9**	**1.3**	**tr**	**1.1**	**1.0**	**0.8**	**3.4**	**0.2**	**3.9**	**tr**	**1.7**
Cubenol	1624	-	-	-	-	-	-	-	-	-	1.3	-
α–Eudesmol	1634	1.2	3.8	1.3	3.3	3.4	2.7	tr	3.3	tr	-	1.0
α–Bisabolol	1656	-	-	-	-	-	-	-	-	-	0.4	-
% Identification		**77.0**	**73.4**	**72.5**	**79.2**	**81.4**	**80.9**	**86.3**	**79.6**	**87.9**	**69.8**	**44.7**

RI: Relative retention indices calculated against *n*-alkanes. - : not present, tr: trace (< 0.05 %); *: determined by mass spectra only.

NB: Sample from February collection was not done due to the lack of plant material.